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THE DISTRIBUTION OF ALKALINE PHOSPHATASE IN VARIOUS TISSUES. By G. BOURNE, MacKenzie MacKinnon Research Fellow of the Royal College of Physicians of London and the Royal College of Surgeons of England. From the University Laboratory of Physiology, Oxford.

INTRODUCTION.

ALMOST identical techniques have been developed independently for the histological demonstration of alkaline phosphatase [Takamatsu, 1939, and Gomori, 1939]. Subsequently Gomori [1941 *a*] published his results in greater detail and also produced a technique for demonstrating acid phosphatase in tissues [1941 *b*]. Kabat and Furth [1941] modified Gomori's technique slightly and gave an account of the distribution of phosphatase in both normal and pathological tissues.

These authors have restricted their descriptions in most cases to the general distribution of the phosphatase in various organs and have given only a cursory description of the intracellular distribution. This study has been carried out with the object of describing the intracellular distribution in greater detail, and special attention has been devoted to the rôle of phosphatase in bone formation and other calcification processes.

METHOD.

The method elaborated by Gomori and by Takamatsu, as described by Gomori, is briefly as follows. Thin pieces of tissue (2 mm. thick) are fixed in 80 per cent. alcohol for 24 hours and then dehydrated through cedar wood oil and benzene (xylene will do as well without the cedar wood oil) and embedded in paraffin. Thin sections (5–7 μ) are cut in the usual way, and the tissue, mounted on slides, has the wax removed and is brought to water and then incubated in a mixture containing sodium glycerophosphate and calcium chloride or nitrate, together with a suitable buffer. The pH of the mixture is approximately 9·4. The phosphatase, which is precipitated *in situ* in the

tissue by the alcohol, splits phosphate from the glycerophosphate. This is trapped immediately and precipitated at the site of phosphatase activity as insoluble calcium phosphate by the free calcium ions in the mixture.

The length of incubation is given as $1\frac{1}{2}$ hours, but a good reaction can be obtained within as short a space of time as half an hour and the sections may be left in as long as 3–4 hours without damage.

After incubation the tissues are rinsed in a 0·5–1 per cent. solution of calcium chloride or nitrate, placed in 2 per cent. cobalt chloride or nitrate for 2 minutes, and then washed thoroughly and placed in a dilute solution of yellow ammonium sulphide for 1 minute. They are then counterstained if desired, and dehydrated, cleared, and mounted in balsam. The sites of phosphatase activity appear black. Kabat and Furth [1941] used a technique which was fundamentally the same as that described above, but they added magnesium sulphate to the substrate mixture (magnesium is a well-known promoter of phosphatase activity [Erdtman, 1927]) and after incubation they stained the tissues by von Kossa's method for demonstrating calcium, thus obtaining a brown colour at the site of phosphatase activity. This method, however, seems much less specific than that of Gomori, because the technique involves the demonstration of the calcium phosphate, newly precipitated by the activity of the phosphatase, and Cameron [1930] has shown that von Kossa's method is specific for neither calcium nor for phosphate. Nevertheless, using adequate controls this latter method is probably satisfactory. The specificity of this technique has been discussed in detail both by Gomori and by Kabat and Furth and need not be repeated here. It should be mentioned, however, that preformed phosphates can be demonstrated by passing a control section through the reagents without incubation in the substrate mixture. This slide acts as a control, and such a preparation should be made for every tissue investigated.

Both Gomori and Kabat and Furth state that this technique can not be applied to adult bone because the process of decalcification destroys the phosphatase. Kabat and Furth, however, found that they were able to remove the small amount of inorganic salts in the bones of a still-born baby with diammonium citrate without affecting the phosphatase reaction.

The present author has found that pieces of bone may be fixed in 80 per cent. alcohol and then incubated with the ester mixture and the calcium phosphate so formed demonstrated by Gomori's cobalt chloride method. Once the cobalt sulphide is formed it is insoluble in the decalcifying agent (trichloracetic acid) and decalcification can therefore be carried out as a last stage. Controls may be made in the same way, omitting the incubation with the substrate. The controls show that the periosteum and endosteum contain appreciable amounts

of preformed phosphates in young animals.   But it is interesting that the true bone itself does not react.   The presence of preformed phosphates could not, of course, be observed in the preparations of Kabat and Furth where decalcification preceded incubation with the substrate mixture.

Needless to say, while the bone still contains its inorganic constituents there is very little penetration by the fluids in which it is placed. This means that it is desirable to use only small pieces of bone, otherwise the endosteum will not stain at all, and in any case only the endosteum and the periosteum and the superficial parts of the bone are likely to give any reaction.   On the other hand, the method is very suitable for investigating the distribution of phosphatase in the repair tissue contained in a small 1 mm. hole previously bored in the bone.   The fluids used penetrate easily into such a hole.

The calcium phosphate precipitated by the enzyme may also be stained by a number of methods used for staining bone (*e.g.* purpurin), none of them very specific.   The present author has used dyes which give *in vivo* staining of bone for demonstrating this calcium phosphate.

The two dyes which have been used are 1 : 2 : 5 : 8 tetra-hydroxy-anthraquinone (kindly sent me by Professor J. C. Brash of Edinburgh) and sodium alizarin sulphonate.   The latter, because of its ready solubility in water, has given better results.   These dyes were added to the incubating mixture and presumably their molecules form a lake with the calcium ions, and when the latter were precipitated by the phosphate liberated from the ester they gave a coloured precipitate of calcium phosphate.   The technique is as follows: Tissues are first fixed for 24 hours in 80 per cent. alcohol, dehydrated, cleared, embedded. Sections, with wax removed, are brought to water and incubated in Gomori's substrate mixture, to which 0·01 per cent. of sodium alizarin sulphonate has been added.   Stronger solutions of the dye are likely to stain all the tissue irrespective of the action of the phosphatase. If this occurs, then the dye concentration is too great and must be reduced.   In general, the weaker the solution and the longer the time of incubation, the better the result.   The time and concentration appear to vary for different tissues.   Bone and kidney have given good results by this method.   Bone, of course, cannot be decalcified after this procedure, which is of use only for macroscopic staining of this tissue.

RESULTS.

The majority of the results here described have been obtained by the application of Gomori's technique to various tissues.   A total of 25 guinea-pigs provided most of the material described, other material is referred to where necessary.

Tongue.—The superficial cells of the filiform papillæ stained darkly

and there were localized positive areas situated more deeply in the epithelium. The cytoplasm and nuclei stained with approximately equal intensity and diffusely. Numerous faintly positive nuclei were present in cells scattered through the epithelium. The tongue muscle and connective tissue were negative.

Stomach.—There was a slight reaction in the serous membrane. There was no reaction in the superficial cells of the mucous membrane, but in the glands about midway between the surface of the mucosa and the submucosa occasional cells could be seen with a slight diffuse staining of the cytoplasm and strongly staining nucleoli. Towards the bottom of the mucosa the walls of capillaries were stained deeply. The endothelial cell nuclei of the capillaries stained more intensely than the cytoplasm. Staining was diffuse and there was no localized staining of the nucleolus. The greatest number of positively staining capillaries were situated at the bases of the glands. Some of the capillaries contained positively staining granules within the lumen. No reaction was given by the muscular coats or the nerve plexuses of the stomach.

Duodenum (Plate I, fig. 1).—There was a strong positive reaction by the epithelium. The cells in the lower parts of the villi and those lining the crypts were positive. The reaction faded away towards the tips of the villi. The reaction was strongest in the brush borders of the cells. In some, the whole of the distal portion of the cell stained diffusely. Lymphocytes (A) which were present among some of the columnar cells had deeply stained nuclei and a completely negative cytoplasm. Most of the nuclei of the epithelial cells were negative or faintly positive and a small number showed a positive staining of the nucleoli. In some of the epithelial cells there was a dark band across the cell about half-way between the nucleus and the distal edge of the cell (not shown in the figure). Then there was a poorly stained area which increased in intensity as the brush border was approached. The most intense reaction was given at the junction with the brush border. The outer edge of the brush border also gave a very strong reaction. If the illustration is examined carefully this double structure of the brush border can be seen. In most cells the phosphatase appeared to be diffused in parts of the cells in which it was distributed, but in some black granules were present also. Brunner's glands and the musculature of the duodenum gave a negative reaction. In some parts of the duodenum the capillaries stained intensely. In some of the capillaries the red blood-cells gave a positive reaction, and many contained black granules within the lumen as well. Both nuclei and cytoplasm of the capillary endothelial wall stained.

Jejunum (Plate I, fig. 2).—The whole of the epithelium lining the villus and crypts was positive. The brush borders of the epithelial cells stained strongly, and most of the cytoplasm of the cells was also

diffusely stained, though less intensely than the brush border. Distribution of phosphatase in the cells was, in general, similar to that of the epithelial cells of the duodenum. The brush border, however, was much wider in the jejunum, and its triple structure (outer thin line of black, broad centre with small reaction, inner part and distal cytoplasm of the cell, black) can easily be seen in the illustration. Nucleoli of the epithelial cells stained strongly (these do not show· very well in the illustration). Amorphous black granules were also distributed through the cytoplasm of some cells and a few showed nuclei with black caps reminiscent of the Golgi apparatus. It is noteworthy that such black-capped nuclei never occurred in cells which showed a strongly staining nucleolus. This result suggests that the black caps may have been due to extrusion of nuclear phosphatase through the cell membrane. The capillaries gave a very slight positive or a negative reaction. The muscle layer was negative.

Colon (Plate I, fig. 3).—In the colon there was a complete absence of staining of the brush borders or of any part of the epithelial cells (A). On the other hand the capillary vessels were stained. The cytoplasm of the capillary endothelial cells was lightly and diffusely stained, but the nuclei gave a much stronger, but diffuse, reaction.

Rectum (Plate I, fig. 4).—The positive reaction of the brush border of the epithelial cells returns in this part of the alimentary tract. In addition to the strong positive reaction given by this part of the cell the distal portion of the cytoplasm stained. There was slight staining of the nuclei. Everything else in the rectum was negative.

SUMMARY OF ALIMENTARY CANAL.

Stomach.	Duodenum.	Jejunum.	Colon.	Rectum.
Epithelial cells of mucosa negative. Capillaries strongly positive.	Deeper epithelial cells positive. Brush border and distal portion of cell positive. Some capillaries positive.	Strong positive reaction by brush border and distal cytoplasm of epithelial cells. Capillaries slightly positive or negative.	Negative reaction by epithelial cells. Positive reaction by capillaries.	Strong staining by brush border and distal portion of cytoplasm of epithelial cells. Capillaries negative.

The two points of greatest interest in this summary are first the presence of phosphatase in the brush borders of cells in those parts of the alimentary tract where most active absorption (at least of glucose) can take place, *i.e.* duodenum, jejunum, and rectum, and the absence of a reaction in the epithelial cells in those parts of the tract which are not important centres of absorption, *i.e.* stomach and colon.

Secondly, the inverse relationship between the staining of the capillaries and the brush borders of the epithelial cells. When the

latter stain, the former give either a slight positive or a negative reaction, and *vice versa*.

Liver and Gall Bladder.—The liver gave practically· no reaction in the guinea-pig—hepatic cells, Kupffer cells, and capillaries appeared negative. The gall bladder (Plate I, fig. 5), on the other hand, gave a strong reaction. The epithelium stained more intensely than any other part. The cells, both cytoplasm and nuclei, were diffusely stained, the nucleus slightly less intensely than the cytoplasm. In addition, particulate masses of black material of varying sizes were present in the distal cytoplasm. The brush borders of the cells stained very intensely. Some nuclei were surrounded by amorphous black granules. The cytological picture given by the cells was one which suggested a secretion of phosphatase by the gall-bladder cells into the bile. In all the sections examined the bile present gave a positive reaction. It is very likely that the phosphatase present in the bile of the guinea-pig comes entirely from the gall bladder because there is no staining of the bile in the bile capillaries of the liver. The bile capillaries of the liver of an adult human being, chicken, and mouse also give negative reaction [Kabat and Furth, 1941], so that it appears that a general function of the gall bladder in at least four species of animal may be the secretion of phosphatase into the bile. There was also a faint staining of the nuclei in the smooth muscle coat of the gall bladder in the guinea-pig.

Salivary Gland (submandibular).—The whole of the salivary gland gave a strong but diffuse staining. Nuclei and cytoplasm of the alveolar cells stained with equal intensity. There was a slight reaction on the part of the control section, indicating that preformed phosphates were normally present in the salivary gland. The salivary glands are probably the chief source of salivary phosphatase.

Spleen.—The Malpighian corpuscles gave a slightly positive reaction. The nuclei of the lymphocytes throughout the spleen gave a faint positive and diffuse reaction.

Mesentery.—Pieces of mesentery examined gave a uniformly negative reaction. Even the capillaries showed no trace of a black deposit.

Muscle.—Voluntary muscle was uniformly negative. Occasionally a faint diffuse staining of the nuclei of the smooth muscle attached to various organs was apparent. Heart muscle (Plate I, fig. 6) from the ventricle gave a slight positive reaction. The capillaries running through the heart muscle, however, gave a strong positive reaction. Both nuclei and cytoplasm of the endothelial cells of the capillaries stained—the former slightly more intensely than the latter. Larger vessels (arterioles and venules) gave no reaction. The control sections showed a slight staining of the endothelial walls of the capillaries, indicating that there were preformed phosphates present.

Trachea and Lung.—The cartilage of the trachea was negative. A

positive reaction was given by the brush borders of the ciliated epithelial cells, and in parts the basal cells gave a diffuse reaction. Some of the deeply lying (submucous) mucous glands gave a positive reaction. In the lung (Plate I, fig. 8) the capillaries gave the strongest reaction, although not all of them were positive. In most capillaries the nuclei of the endothelial cells stained very much more intensely than the cytoplasm. They always stained diffusely. Some of the capillaries showed black granules in the lumen. Some of the alveolar cells also gave diffuse staining of the nuclei and there were also patches of alveolar cells in which the whole cell stained faintly and diffusely. The same type of patchy diffuse staining was shown by the cells of the bronchus with the exception that many of the latter cells had positively staining nucleoli. In the cartilage present in some of the larger bronchi both cartilage cells and the matrix were negative.

Nervous System.—No nerve cells gave a positive reaction, but in the spinal cord there appeared to be a slight reaction by the nerve fibres in the outer parts of the horns of grey matter. The characteristic feature of the cerebellum (Plate I., fig. 7) and cerebrum and the spinal cord was the strong staining of the endothelial walls of the capillaries. The nuclei and cytoplasm of these cells appeared, in general, to stain with equal intensity. With the exception of the reaction in the spinal cord already mentioned, all the other components of these three tissues gave a negative reaction. The optic nerve was completely negative.

Skin (Plate II, fig. 1).—In the skin there was no reaction by the subepidermal connective tissue. In the epidermis only the stratum granulosum cells gave any reaction, and this was a weak one. The hair follicles, however, stained strongly. There was only a slight reaction at the bottom of the follicle, but the reaction was intense in most of the central part of the hair sheath. The region of the hair sheath near the epidermis was devoid of reaction. The sebaceous glands stained strongly. Nuclei and cytoplasm of all positive cells appeared to stain diffusely and no granules could be observed. There was no staining of the nucleoli.

Some hairs gave a fairly intense staining, but staining of equal intensity was found in the controls. The hair follicles stained in the same manner, but much less intensely in the controls than in the incubated section.

Male Reproductive Organs.—The testis gave a strong reaction in the capillaries. There appeared to be a general staining of the testis, including both the interstitial and the generative cells. All chromosomes in cells undergoing maturation division gave a positive reaction, but it was not any more intense than in any of the other cells. Some of these cells contained black granules, but the fixation of this organ by the alcohol was so bad that it was impossible to observe accurate detail. There was no reaction in the vas deferens or the vesicula

semenalis.   The penis contained a few positive capillaries, but all else was negative.

Urinary System.—In the ureter (Plate II, fig. 2) the distal portions of the superficial cells of the epithelium were almost devoid of phosphatase.   Proximal portions stained strongly.   The cytoplasm of these cells and of those lying deeper in the epithelium was diffusely stained.   The nuclei of all the epithelial cells gave a stronger reaction than the cytoplasm, and the reaction was of greater intensity in the nucleoli.   Some cells showed scattered granules in the cytoplasm.   In the bladder the superficial cells gave no reaction, but the deeper cells gave a diffuse reaction in both nuclei and cytoplasm.   The epithelium lining the kidney pelvis also gave a strong reaction similar to that found in the ureter.   The kidney (Plate III, figs. 7 and 8) has been examined in both the guinea-pig and the rat.   In both animals the glomeruli did not stain, and the most intense reaction was given by the cells of the convoluted tubules.   In the rat the whole cell stained, but the most intense reaction was localized in the outer border of the cell, and particularly in the brush border.   In many of the guinea-pig kidneys examined the reaction was restricted to the distal fifth of the cell.   There was staining of the nuclei in many cells but the reaction was diffuse, and in very few nuclei was there any evidence of nucleolar staining.

Endocrines.—The thyroid gave no reaction.   The anterior pituitary gland (Plate II, fig. 3) gave a positive reaction.   Some groups of cells seemed to react more strongly than others, but they could not be reconciled with any particular cell type.   There were present two types of nuclei, those which possessed a number of large coarse black granules (B) and those which showed no staining of the nucleoli, they stained simply a diffuse dark brown (A).   It is known that in both the chromophil and basophil cells of the anterior pituitary there are two kinds of nuclei, those with a coarse chromatin and those with a fine chromatin network.   It is probable that the two types of phosphatase staining in the nuclei were an expression of this original morphological difference.   In the adrenal of the guinea-pig (Plate II., fig. 4) the greatest reaction was given by the cells.   Those of the zona glomerulosa stained deeper than those of any other zone.   The inner part of the cortex gave no reaction at all.   The distribution of the phosphatase was very similar to that of the distribution of the fat and cholesterol (with the exception of its high concentration in the zona glomerulosa).   In this zone (glomerulosa) the nuclei stained diffusely and the cytoplasm contained numerous rodlets and filaments.   Towards the centre of the zona fasciculata there were two types of cell, one type with diffuse staining nuclei and another with nuclei which contained an obvious stained reticulum.   These two types of cells were not found in all the specimens of guinea-pig adrenal examined.   Some zona

fasciculata cells possessed dark granules between the "lipoid" droplets. There was some staining of the endothelial cells of the blood sinuses. The rat adrenal (Plate II, fig. 5) gave an entirely different picture from this. All the nuclei of all the cells stained faintly and diffusely. The most striking feature of the rat adrenal was the way the capillary network of the gland was picked out by the reaction. Nuclei and cytoplasm of the endothelial cells of the capillaries stained heavily and diffusely. The nuclei of some of the endothelial cells were very much swollen, and in some cases they almost blocked the sinuses. Many of the capillaries contained black granules in the lumen.

Bone Marrow.—The nuclei of all the bone-marrow cells were positive. It was difficult to distinguish all the various types of cells, but there seemed to be a greater staining in the primitive granulocytes than in the primitive red cells. Megaloblasts gave a stronger reaction than other primitive red cells. The capillaries gave practically no reaction.

Effect of Vitamin C Deficiency on the Phosphatase Reaction of Soft Tissues.—The kidney and the adrenal were used for these experiments. Six guinea-pigs were placed on a scorbutic diet for two weeks and were given graded doses of vitamin C by injection. One animal received 4 mg. of vitamin C and five others received 2 mg., 1 mg., 0·5 mg., 0·25 mg., and no vitamin C respectively. Sections of kidney and adrenal were treated in an identical manner but showed no difference in the intensity of their phosphatase reactions. Similar results of a quantitative nature for soft tissues have been obtained independently by Harrer and King [1941] and Gould and Shwachman [1942].

Bone.—In normal femur, humerus, and parietal the phosphatase reaction appears to be restricted to the periosteum and the endosteum and to such superficially placed osteocytes and their processes as are reached by the substrate mixture and the subsequent reagents. The inorganic matter of the bone does not react with the cobalt-chloride and ammonium-sulphide reagents. The reaction in the periosteum appeared due to the osteoblasts and the capillaries. In some preparations some of the collagen fibres stained too.

An investigation was carried out into the distribution of phosphatase at various stages after boring a hole in both femora. The hole was bored with a 1-mm. dentist's twist drill and the operation was carried out aseptically. It has been shown by Roche and Fillipi [1938 *a* and *b*] that when a bone is injured there is an increase in the phosphatase content of the bones in the whole of the skeleton. This suggests that some hormone-like substance is liberated as a reault of the injury (it is of interest that Williams and Watson [1941] have shown that corticosterone increases the phosphatase activity of bones). It may be of value to record at the same time the fact, mentioned in one of their early papers by Wolbach and Howe [1926], that if the bone of the leg

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Urinary System.—In the ureter (Plate II, fig. 2) the distal portions of the superficial cells of the epithelium were almost devoid of phosphatase. Proximal portions stained strongly. The cytoplasm of these cells and of those lying deeper in the epithelium was diffusely stained. The nuclei of all the epithelial cells gave a stronger reaction than the cytoplasm, and the reaction was of greater intensity in the nucleoli. Some cells showed scattered granules in the cytoplasm. In the bladder the superficial cells gave no reaction, but the deeper cells gave a diffuse reaction in both nuclei and cytoplasm. The epithelium lining the kidney pelvis also gave a strong reaction similar to that found in the ureter. The kidney (Plate III, figs. 7 and 8) has been examined in both the guinea-pig and the rat. In both animals the glomeruli did not stain, and the most intense reaction was given by the cells of the convoluted tubules. In the rat the whole cell stained, but the most intense reaction was localized in the outer border of the cell, and particularly in the brush border. In many of the guinea-pig kidneys examined the reaction was restricted to the distal fifth of the cell. There was staining of the nuclei in many cells but the reaction was diffuse, and in very few nuclei was there any evidence of nucleolar staining.

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in the animal receiving 0·5 mg., and no staining in the scorbutic animal. Confirmation of these results were also obtained from the other femora which were passed through cobalt chloride and ammonium sulphide in the usual way. After decalcification and sectioning, microscopic observation showed that there was very much less deposition of black cobalt sulphide in the scorbutic than in the non-scorbutic animal. There was also less deposit of black material in the periosteum of the femora and the humeruses.

These results indicate that there is less phosphatase in regenerating and intact bone in scorbutic than in normal animals. While these experiments were being carried out a paper was published by Gould and Shwachman [1942] in which it was found by quantitative methods that there was a decrease in the amount of phosphatase in the bone in scurvy.

Detailed observations were made of the distribution of phosphatase in the tissue filling the holes in the various femora (Plate II, fig. 6). In one of the animals receiving 2 mg. vitamin C, the periosteum near the hole gave an intense reaction (P), due chiefly to the deep staining of the osteoblasts and the capillaries. The osteoblasts stained diffusely, and cytoplasm and nucleus stained with equal intensity. Some of the periosteal cells near the cartilage which had formed (c) gave a very strong reaction. It was observed that when the cartilage cells were hypertrophied and had little matrix around them they often stained with great intensity. In some cases when they were surrounded by quantities of matrix which had given a positive phosphatase reaction the cell itself contained very little of the enzyme, but a dense deposit could be seen at the boundary of the cell capsule and the matrix. The appearance was as if the cartilage cell (apparently derived either from a fibroblast or an osteoblast of the periosteum) had charged itself with phosphatase (probably by synthesis) and then as it produced and excreted the cartilage matrix it excreted the phosphatase with it. Other specimens of cartilage were found, however, in which the cartilage cells embedded in a matrix containing phosphatase, themselves contained a high concentration of enzyme. In those animals receiving the smaller doses of vitamin C, fibroblasts were seen which were outlined with a black border, presumably due either to the synthesis of phosphatase there by the fibroblast or its absorption from the medium. Other fibroblasts were found with one process stretched out until it had become an elongated thread which had stained black. Most of the fibres in the hole were found to give a positive phosphatase reaction, and these results indicate what may be the method of formation of these fibres—that is, the "spinning" of a phosphatase impregnated film of protein from the surface of the fibroblast. These results are in keeping with Danielli's [1942] suggestion for the origin of tissue fibres.

The osteoid trabeculæ were composed of darkly staining fibres

of a scorbutic guinea-pig is injured there is a temporary repair process initiated in the teeth, as if, according to Wolbach, injury to the bone caused the production of antiscorbutic substance in the body. It seems that this phenomenon was probably due to the increased phosphatase activity of the bony tissues brought about by the injury to the bone.

In view of Roche and Fillipi's results, pieces of parietal bone and a humerus were taken from an animal, the femora of which had been bored as described. Animals were killed 3 hours and 24 hours after the operation of boring the hole. The pieces of parietal and the humerus were fixed in alcohol for 24 hours, brought to water and incubated for 2 hours in Gomori's substrate mixture, then treated with cobalt chloride, etc. No difference could be seen between the phosphatase reaction of any of the bones, compared with those from a normal, either macroscopically or microscopically. So if there is an increase in the phosphatase content of the skeleton after injury to the bone, either the injury made in these experiments was not severe enough or its detection requires a more accurate quantitative technique.

There was no immediate concentration of phosphatase in the clot filling a hole bored in the femur (3 hours after operation) nor was there any observable increase after 24 hours. By 24 hours there was an active migration of fibroblasts coming both from the marrow and the periosteum. They gave a negative phosphatase reaction. On the other hand, some cells in the marrow, the nature of which could not be ascertained with certainty, contained a dense aggregation of black granules.

Attempts were next made to see if vitamin C deficiency inhibited the appearance of phosphatase in the hole.

It is conceivable that this would be so, because it has been shown that the number of bony trabeculæ forming in such a hole is directly proportional to the amount of vitamin C the animal receives [Bourne, 1942] and it is unlikely that there would be a considerable increase in the amount of phosphatase present until active formation of the trabeculæ was beginning. For these experiments six guinea-pigs were taken and placed on a scorbutic diet; they were given supplementary doses of vitamin C of the same amounts as described above. At the end of one week of this régime a hole was bored in each femur and at the end of another week the animals were killed. Their femora were fixed in alcohol and subsequently incubated with Gomori's substrate mixture, and finally decalcified after treatment and sectioned. One femur of an animal on 2 mg. of vitamin C, one of an animal on 0·5 mg., and one of an animal receiving no vitamin C were incubated with Gomori's reagent containing 0·01 per cent. of sodium alizarin sulphonate. After 1 hour's incubation there was a strong pink-purple staining of the tissue filling the hole in the 2-mg. animal, a slight staining

Mid-albumen Region.—The cuticular margins of the epithelial cells gave a positive reaction in both A and B. All the other tissues were negative. In A the blood-vessels were positive and in B they were negative.

Isthmus near Uterus.—In both A and B there was a strong positive reaction of all blood-vessels. The epithelium was darker than the rest of the section. The cuticular borders of the epithelial cells were darkened.

Uterus.—A. All the blood-vessels stained. The reaction was given both by the inner part of the wall and the endothelium. The red blood-cells present in the blood-vessels stained slightly. There was a slight darkening of the epithelium and the greatest intensity was given by the brush borders.

B (Plate III, fig. 3). The epithelium stained darker than any of the other tissues. The cuticular margins stained deeply. The nuclei of the epithelial cells contained granules (positive nucleoli). The phosphatase in the cytoplasm appeared diffused throughout the cell. Mucous secreting cells between the ciliated cells contained very little phosphatase. Tubular glands in the corium (Plate III, fig. 5, and "A" in Plate III, fig 3) showed positive granular nuclei, and the outermost portion of the distal regions of the cells of these glands contained numerous granules of positive material.

Conrad and Scott [1938] state that shell deposition in the egg begins as soon as the egg reaches the uterus, therefore one would expect the uterus to contain more phosphatase than any other part of the oviduct, and this was the case, but there was not the intensity of reaction that one would have expected from a tissue capable of depositing such large quantities of calcium in the relatively short time required to produce an egg shell. Since a large percentage of the egg shell is calcium carbonate rather than phosphate, it seems that phosphatase may not play an important part in the mechanism of its deposition; the present results support this suggestion.

A further investigation was made of marine animals which produce a shell. One would expect a rather similar result to that obtained for the fowl oviduct, since a rather large proportion of the shell of such animals is also calcium carbonate.

A specimen of prawn (*Leander*) had a chitinous shell which appeared to have a considerable affinity for cobalt chloride, and it was therefore impossible to be certain of the presence of phosphatase. The membrane immediately beneath the shell gave only a slight reaction. The prawn possesses a lightly calcified cuticle, and that of a highly calcified form; a lobster (*Homarus*) was therefore examined. The cells of the membrane beneath the shell showed positive nuclei but the cytoplasm was negative. These animals shed their exoskeletons periodically, and the formative membrane was probably inactive at the stage at which this

(Plate III, fig. 1), and in many cases appear to be aggregated around capillaries which gave a positive phosphatase reaction. In one specimen it was seen that fibroblasts near the boundary of the marrow and the tissue filling the hole gave a very slight or negative reaction—some dark granules were observed in their nuclei. In the tissue of the hole the fibroblasts were much darker, indicating an increase in the amount of phosphatase present in the cell. Practically all the fibres in the hole gave a positive reaction (Plate II, fig. 7), and even in the control sections many of them were dark, indicating the presence even at this early stage of preformed phosphates, but not apparently of true bone salt, which does not react with the cobalt chloride and ammonium sulphide used on the controls. Microscopically there was scarcely any phosphatase reaction in the holes in the femora of the scorbutic animals, and even the periosteum gave a very much reduced reaction.

In view of the importance of the question of the relationship of phosphatase to calcification and bone formation, a relationship which is by no means universally accepted, *e.g.* Lichtwitz [1942] in his recent book states "there seems very little probability that phosphatase plays a part in the process of calcification of bones," it was decided to investigate the distribution of phosphatase in the tissues of other animals which are associated with calcification processes.

The first animal examined was the laying hen. Peterson and Parrish [1939] have shown that the serum phosphatase of the hen increased by 30 per cent. during the period of shell formation, and it seemed desirable therefore to examine the oviduct of the hen during the laying period. Naturally it is at present difficult to obtain, and undesirable to use, a large number of laying hens, but it was possible to obtain two which were approaching the end of the laying period. One was killed 36 hours after having laid a well-shelled egg and one was killed while there was a soft-shelled egg in the uterus and other smaller ones in the upper part of the oviduct. The oviduct of the fowl, as can be seen from Richardson's [1934] illustration, can be divided into chalaziferous region near the ovary, followed by an albumen region, an isthmus, and a uterus. Sections were examined through these four regions in both fowls. Duplicate preparations were made by Gomori's technique and by the alizarin modification previously described in this paper. The best preparations of the uterus were obtained by this latter method. The tissues from a hen which had laid an egg 36 hours previously will be referred to as A and those from a hen which contained eggs in the oviduct as B.

Chalaziferous Region.—A. Most of the blood-vessels gave a positive reaction. Everything else, including the epithelium, was negative.

B. All blood-vessels were negative. There was a general but slight darkening of the epithelium. The cuticular margins of the epithelial cells gave the strongest reaction.

this is so, then we have an explanation of the presence of phosphatase in the mantle edge.

These results with *Mytilus* have also been duplicated with the Gastropod Mollusc *Calliostoma*.

DISCUSSION.

Phosphatase has been known for some time to be widely distributed in the body, both in tissues which ossify and in those which do not ossify. Although it is characteristic of this phosphatase that it can split glycerophosphate and other monoesters of phosphoric acid at a high pH (about 9·0), it is not known whether all these phosphatases are the same. Bodansky [1937], for example, showed that the activity of bone and kidney phosphatase was retarded by bile acids and that the intestinal phosphatase, as might be expected, was not. King and Hall [1930] have shown also that the phosphatase activity of bone and kidney of hens was reduced by feeding irradiated ergosterol. On the other hand, Page and Reside [1930] found that in dogs irradiated ergosterol reduced the phosphatase content of kidney and intestine. The fact that Huggins [1931 *a* and *b*, 1933] found that pieces of urinary and gall-bladder epithelium transplanted into the rectus muscle sheath produced bone, suggests that the phosphatase of these organs are identical. Nevertheless that of the gall bladder is obviously not seriously affected by the presence of bile salts, and therefore it is similar to that of the intestinal epithelium. These results all suggest an identity of the phosphatases which split monoesters of phosphoric acid.

The presence of phosphatase in both nuclei and cytoplasm is of interest, and raises once more the question of identity of the phosphatase found in these two sites, and in particular of the function of the nuclear phosphatase. A so-called nucleotidase had been extracted from animal tissues by Levene and Dillon [1930] and others, and Folley and Kay [1936] consider this to be identical with the alkaline phosphatase of bone, kidney, etc. It seems possible that the alkaline phosphatase which is present in the nuclei of various cells is associated with the nucleic acid-nucleotide metabolism of the cell and with the ebb and flow of nucleotides to and from the nucleus and cytoplasm [see White, 1942]. It is of interest in this connection that phosphatase is present in the chromosomes of dividing cells and that in the chromosomes of mitotic tissue culture cells practically all the phosphatase of the cell is located in or around the chromosomes [Willmer, 1942]. It is of interest that Horning [1942] has stated that in the division of tissue culture cells practically all the mineral salts of the cell are aggregated in the chromosomes.

The distribution of phosphatase in the different parts of the alimentary tract agrees with those obtained by other workers, although

specimen was examined; the membrane of an animal actively engaged in the process of forming a new skeleton would probably give a better result. It was thus decided to use animals in which there was a more continuous formation of shell. Two representatives of the Mollusca, the mussel (*Mytilus*), and a Gastropod (*Calliostoma*) were examined. Even in the Mollusca the secretion of the shell takes place in cycles [Cooke, 1895], but there is a greater possibility of striking an active stage of shell secretion in these animals. The mantle of these animals is responsible for the production of the shell, and the most active region is believed to be the mantle edge, and it is by constant deposition of shell from this part of the mantle that the shell grows in size. Manigault [1939 and 1941] found a direct correlation in Molluscs between liver, mantle, and blood phosphatase and calcium precipitation of the shell.

In *Mytilus* (Plate III, fig. 6) it was found that the mantle edge gave a very strong reaction, and a similar reaction but of lesser intensity was given by the control, indicating the presence of preformed phosphate. In the phosphatase preparations the distal portions of the cells stained a deep diffuse black or brown and were filled with brown granules. A section of the mantle edge without incubation showed the presence of a number of black or brown granules, and in a section which was untreated, even by the control reagents, there were yellow and light brown granules in the cells. These granules were aggregated in the region of the Golgi apparatus, and in many cells they were so abundant as to fill most of the distal region of the cell. It is probable that a good deal of the brownish yellow colouring of these granules in the control preparations is due to the natural pigments contained in them. Gatenby and Duthie [1932] and Gatenby and Hill [1933] have found similar coloured granules in the mantle edge of *Helix aspersa*. In white specimens of *Helix pomata* they found that similar granules were present but that they were uncoloured. These authors also found a tendency for the granules to be aggregated in the region of the Golgi apparatus, that they were insoluble in fat solvents (they are present in *Mytilus* after fixation in 80 per cent. alcohol), and that they went black after treatment with Da Fano' Golgi technique. This technique involves the use of silver nitrate, and this result suggests, therefore, that these granules contain calcium phosphate (see von Kossa's test, though this is not necessarily specific). At any rate it seems likely that they represent granules of shell substance about to be secreted. If they are composed of calcium phosphate, then this suggests that the shells of molluscs should contain considerable quantities of this substance. Pelseneer [1906], however, states that in most molluscs the shell contains only about 1–2 per cent. of calcium phosphate. On the other hand, Plate [1922] claims that the calcium of the mollusc shell is secreted first as the phosphate and that it later changes to the carbonate. If

tion does not advance us much further in the consideration of the fundamental relationships between the two substances. This problem needs much more experimental work before it can be profitably discussed.

SUMMARY.

1. Further details of the staining of various organs by Gomori's phosphatase technique have been given.

2. A modification of the technique which involves adding sodium alizarin sulphonate to the substrate mixture has been described.

3. A description has been given of the distribution of phosphatase in regenerating bone.

4. It has been found that phosphatase does not appear to be associated with the calcification of the hen's egg.

5. Phosphatase appears to play some part in the secretion of the shell of certain molluscs.

6. The intensity of phosphatase staining of kidney and adrenal was found to be undiminished in scurvy, but the periosteum and regenerating bone were found to stain less intensely than normal in scorbutic animals.

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neither of them mentions the staining of the brush border in the epithelial cells of the rectum.

Magee and Reid [1931] originally showed that sugars were more efficiently absorbed in the presence of phosphate, and it was shown later by Lundsgaard [1933 a and b] that phloridzin, which inhibits phosphorylation processes, also inhibited the absorption of glucose from the intestine. This conception was later expounded by Verzar [1936]. Sugar, to be absorbed, must first pass through the brush borders of the epithelial cells. Results obtained from this study and those of Gomori, and of Kabat and Furth, show that the brush borders of the cells in the small intestine are heavily impregnated with phosphatase. It is not known for certain whether the phosphorylation of the sugars is actually carried out in the lumen of the intestine by phosphatases secreted there from the epithelial cells or coming from the bile (there is a considerable amount of phosphatase present in faeces), or whether the sugar is first adsorbed on to the surface of the cell or absorbed into the brush border, phosphorylated, and then passed into the interior of the cell. It is of interest that Horning [1942] has noted the aggregation of mineral salts at the borders of intestinal epithelial cells. The fact that glucose can be absorbed from the rectum and that the epithelial cells here contain phosphatase in the brush borders is of interest.

Kabat and Furth [1941] discuss Lundsgaard's theories in regard to sugar resorption by the kidney. Once again the distribution of phosphatase is in the brush borders (of the convoluted tubule cells). On the other hand, it is difficult to appreciate the significance of the localization of the phosphatase in the margins of the cells of the trachea unless perhaps the enzyme is associated with the phosphorylation processes involved in the movement of cilia. The ciliated cells of the fowl oviduct also have phosphatase localized in the cell margins.

The wide distribution of phosphatase in the endothelial cells of the vascular system, commented on by Gomori, and by Kabat and Furth, is one of the most striking results which have emerged so far from the histological studies of the distribution of phosphatase. We must agree with Kabat and Furth, however, that it is impossible to tell whether these results indicate that phosphatase is secreted into the blood by the endothelial cells or whether they absorb it from the blood.

The disposition of phosphatase in regenerating bone lends further support to Robison's original conception that it is associated with bone formation, and from the results obtained from the mollusc it appears to play some part in other types of calcificatory phenomena also.

Since the phosphatase activity of bone is decreased in scurvy it is of interest to consider what relationship may exist between vitamin C and the enzyme. There is, however, little direct evidence. Giri [1939] has suggested that there is a balance between vitamin C, copper, and glutathione which regulates phosphatase activity, but this sugges-

Fig. 4. Epithelium of rectum of guinea-pig. Strong staining of brush border and distal portion of cytoplasm.   × 500.

Fig. 5. Gall bladder epithelium of guinea-pig. Intense staining of brush border. Faint staining of nuclei. Positive staining of bile on the right. × 500.

Fig. 6. Positive capillaries in heart muscle of guinea-pig.   × 250.

Fig. 7. Positive capillaries in cerebellum of guinea-pig.   × 250.

Fig. 8. Lung of guinea-pig. The black dots are the strongly staining nuclei of the endothelial cells of the capillaries.   × 500.

PLATE II.

Fig. 1. Skin of guinea-pig. Strong staining of hair follicles. Staining of hairs spurious. Positive reaction by stratum granulosum of skin.   × 25.

Fig. 2. Epithelium of ureter of guinea-pig. Strong staining of nuclei. × 625.

Fig. 3. Anterior pituitary of guinea-pig. Two types of nuclear staining. Diffuse type (A); granular type (B).   × 625.

Fig. 4. Adrenal of guinea-pig. Strong staining of peripheral cells. Nuclei positive.   × 75.

Fig. 5. Adrenal of rat. Strong positive reaction by capillaries. Slight staining of nuclei of cells.   × 75.

Fig. 6. Repair tissue in hole in guinea-pig femur after one week. Periosteum (P). Cartilage (C). Mass of fibrous tissue in hole (E). Developing trabeculæ (T). Marrow (M).   × 75.

Fig. 7. H.P. fibres in the hole (fig. 6).   × 625.

PLATE III.

Fig. 1. H.P. trabeculæ in hole (see Plate II, fig. 6). Osteoblasts (O) with positive nuclei.   × 125.

Fig. 2. Periosteal trabeculæ forming near the hole (Plate II, fig. 6).   × 25.

Fig. 3. Uterus of fowl with soft-shelled egg in uterus. Staining of brush border. Staining of the inner portions of the distal cytoplasm of tubular gland cells in the corium (A).   × 250.

Fig. 4. Cartilage near the hole (see Plate II, fig. 6). Positive cartilage cells, Some of matrix positive.   × 625.

Fig. 5. Uterus of fowl (fig. 3). Tubular glands in corium. Stained by alizarin modification. Positive gland cell nuclei and staining of inner borders of cells.   × 625.

Fig. 6. Mantle edge of *Mytilus*. Distal portions of cells of granules and phosphatase. At "A" aggregation of granules in the Golgi region may be seen.   × 500.

Fig. 7. Kidney of guinea-pig. Gomori's technique. Reaction localized in distal edges of convoluted tubule cells. Glomeruli negative.   × 40.

Fig. 8. Kidney of guinea-pig. Alizarin added to substrate mixture. Staining fainter but same distribution as in fig. 7.   × 40.

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EXPLANATION OF PLATES.

All the tissues figured in these plates show the results of the application of the phosphatase reactions.   In no case has any other stain been used.

PLATE I.

Fig. 1. Epithelium of duodenum of guinea-pig, showing staining of inner and outer edges of brush border.   In some cells staining of outer portions of distal cytoplasm present.   Nuclei of epithelial cells faintly stained.   Nucleus of lymphocyte in the epithelium (A) gives a much stronger reaction.   × 500.

Fig. 2. Epithelium of jejunum of guinea-pig.   Strong staining of distal cytoplasm of cells and of outer edge of brush border.   × 500.

Fig. 3. Epithelium of colon of guinea-pig.   Strong staining of underlying capillaries.   Negative reaction by epithelial cells (A).   × 500.

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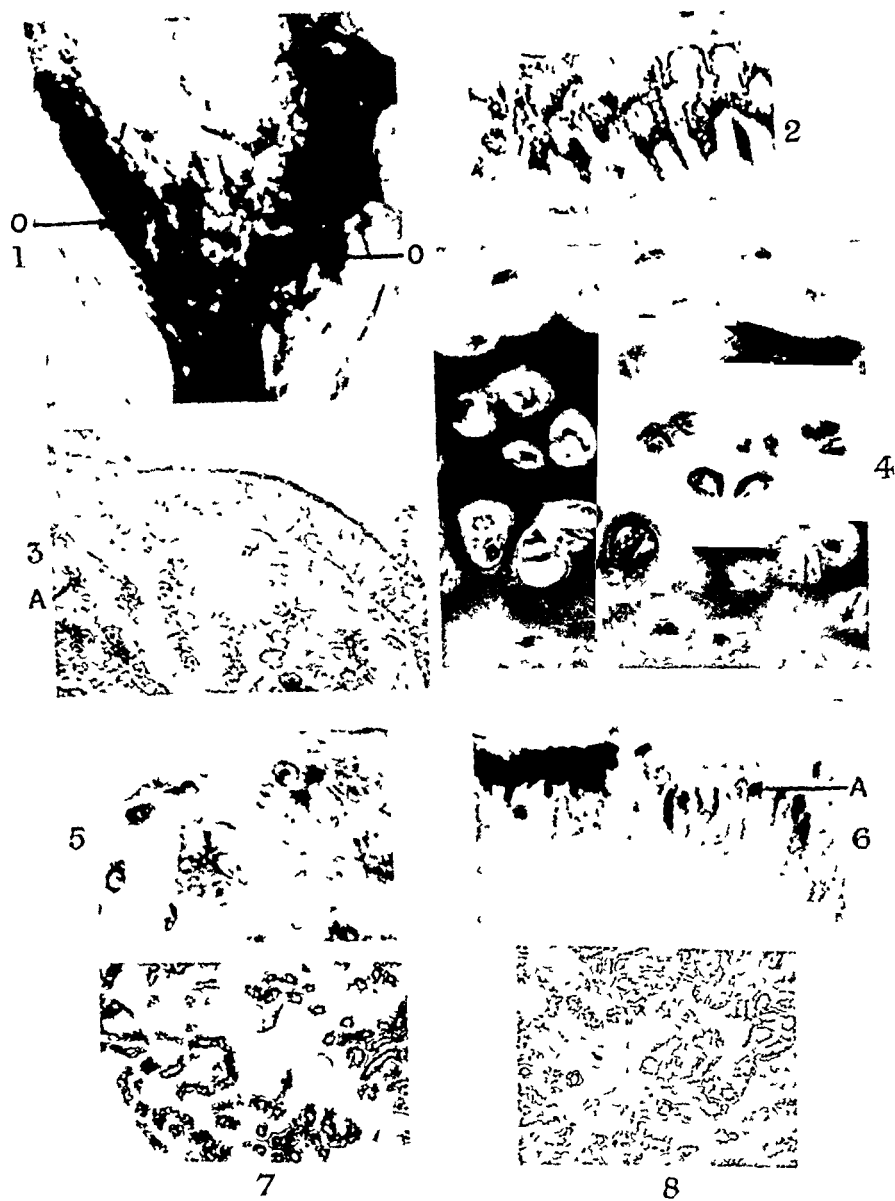
G. BOURNE, "The Distribution of Alkaline Phosphatase in Various Tissues"

A STUDY OF THE DISTRIBUTION OF SULPHANILAMIDE.

By Frank Alexander. From the Department of Pharmacology, University of Edinburgh.

The therapeutic effect of a drug such as sulphanilamide depends on the presence of an adequate concentration of the drug at the site of action; the latter is governed by the way in which the drug is distributed in the body. Marshall *et al.* [1937] showed that, in the dog, sulphanilamide was evenly distributed through all the tissues, excepting bone and fat, four hours after oral administration. From these results it may be deduced that the concentration of sulphanilamide in the tissues is equal to its concentration in the blood. However, Hansen [1940] found that in the blood the concentration of sulphanilamide was higher in the corpuscles than in the plasma, and Riser and Valdigue [1939], using dogs, found that the concentration of this compound was higher in the brain and temporal muscle than in the blood. Marshall's experiments were carried out with dogs, and he found that these animals differed from most others in that they did not acetylate sulphanilamide. The object of the present investigation was to study the distribution and metabolism of sulphanilamide in animals which acetylate this compound.

METHODS.

Sheep, rabbits, and mice were used in this investigation. A 0·5 gm./100 ml. solution of sulphanilamide in saline was injected intravenously in sheep. They were placed in metabolism cages so that the urine could be collected. Blood was withdrawn from the sheep at fixed intervals, and at the same time the cage was well washed, the washings being made up to a known volume. The same solution was injected intravenously in rabbits which were then placed in metabolism cages. At intervals after the injection, groups of rabbits were killed by stunning and bleeding to death and their tissues analysed. The same solution was injected intravenously in mice which were then placed in individual metabolism cages constructed from Buchner funnels. The mice were killed, the cage washed, and the sulphanilamide in the whole mouse and its excreta determined.

Estimations of the sulphanilamide content of the blood and urine

TABLE II.

Sheep No.	Dose, mg./kg.	Time, hours.	Blood conc. mg./100 ml.		Amount remaining in body.	Distribution volume.	Body wt.
			Free.	Total.	mg.	Litres.	kg.
358	68·7	1	5·40	7·35	1187	16·1	
		6	3·20	4·24	1040	24·5	18·2
		24	0·24	0·40	831	207·7	
359	49·3	1	5·54	6·00	1064	17·7	
		6	2·28	3·20	834	26·0	22·7
		24	0·04	0·48	460	95·7	
361	90·0	1	9·84	12·40	2160	17·4	
		5	5·80	8·20	1850	22·7	25·5
		24	1·00	1·90	1570	78·4	
360	41·0	1	4·84	5·54	1009	18·0	
		5	2·86	4·00	869	21·7	26·0
		24	0·40	0·50	483	96·6	
394	163·9	1	17·60	19·20	4250	20·2	
		5	9·10	12·00	3600	30·0	26·0
		24	2·00	6·52	2237	34·3	
Rabbit No.							
8	57·1	3	1·20	2·53	73·6	3·0	1·7
9	53·3	6	0·50	1·22	76·0	5·6	1·5
10	76·9	24	0·52	0·64	34·6	5·4	1·3
7	51·4	48	0·00	0·50	65·5	13·1	1·8

TABLE III.—RECOVERY OF TOTAL SULPHANILAMIDE FROM THE URINE.

Animal.	Time, hours.	Total Drug recovered from excretions.	Dose, mg.
		Per cent.	
Sheep . .	72	80·8	1120
	96	56·4	1250
	96	41·5	2250
	96	61·2	1062
Rabbit . .	72	66·9	100
	72	50·4	100
	96	78·6	100

were made by the method of Bratton and Marshall [1939].   The tissues
were analysed by grinding weighed quantities of minced tissue with
four volumes of 15 per cent. trichloracetic acid, allowing to stand for
at least one hour, then adding 1 ml. of 4N hydrochloric acid to each
20 ml. of the mixture.   The whole mixture was filtered and the
estimation made on the filtrate by the method of Marshall and
Bratton.

RESULTS.

Table I shows the recovery of known amounts of sulphanilamide
added to various tissues.   Table II shows the concentration of free
and total sulphanilamide in the blood, determined at intervals after
the injection.   The amount of drug remaining in the body is calculated
by subtracting the amount excreted in the urine from the amount
administered; it is assumed for this calculation that that excretion
is only by the kidney and that none of the drug is destroyed.   The
volume of distribution is calculated from the formula:

$$\frac{\text{Volume of}}{\text{distribution}} = \frac{\text{Amount of drug remaining in the body (mg.)}}{\text{Concentration of total drug in the blood (mg./litre)}}$$

The volume of distribution may be defined as the volume of fluid
through which the amount of drug in the body would be evenly distri-
buted if the concentration in that fluid was the same as that in the
blood.

TABLE I.—RECOVERIES OF ADDED QUANTITIES OF SULPHANILAMIDE.

Tissue.	Sulphanilamide added. mg./100 gm. tissue.	Amount recovered. mg./100 gm. tissue.
Liver . . .	1·00 mg./100 gm.	0·98 mg./100 gm.
Liver . . .	1·00 mg.	1·02 mg.
Kidney . . .	1·00 mg.	0·97 mg.
Minced mouse . .	2·00 mg.	1·92 mg.
Minced mouse . .	2·00 mg.	2·02 mg.
Minced mouse . .	2·00 mg.	2·04 mg.
Minced mouse . .	1·00 mg.	0·95 mg.

From Table II it will be seen that although the values obtained
for volumes of distribution one hour after the injection may be explained
on the hypothesis of an even distribution of the drug through the body
water, the values obtained at longer intervals cannot be accounted for
on this theory.   This being the case, either the drug is not distributed
evenly through the body water or it is changed into a form which is
not estimated by diazotization.

being retained in the tissues, the possibility of the fixation of the drug by the tissues was then investigated. The results are shown in Table IV. It will be seen that the concentration in the liver and kidneys is higher than in blood. The concentration in the kidney is very high during the first six hours, this is probably due to some urine in which the drug is concentrated being included with the tissue. Table V shows the ratio of concentration of drug in the tissues to the concentration in the blood; with the liver this figure increases with time.

As the amount of sulphanilamide retained by the tissues was too small to account for the difference between the dose given and the
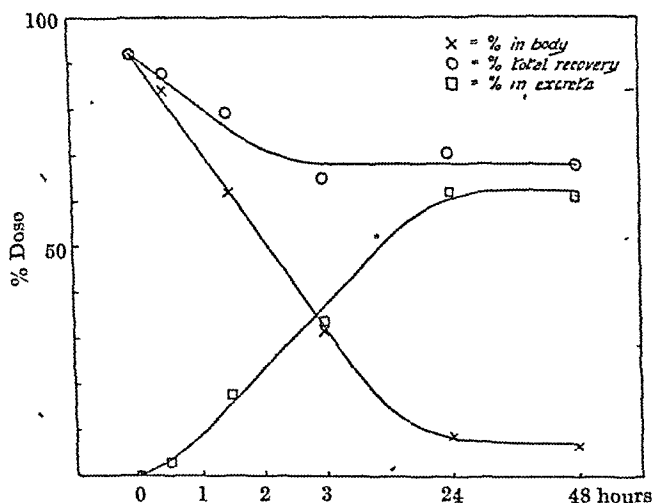
Fig. 1.—Relationship between percentage recovery and time.

quantity recovered from the urine, the possibility of the substance being changed in the body to a form not estimated by diazotization was next investigated.

Mice, injected intravenously with a solution of sulphanilamide, were placed in individual metabolism cages. They were killed at intervals and the total quantity of sulphanilamide in the mouse and in the excreta estimated. The results obtained are shown in Table VI. The validity of the method was checked by killing a group of mice immediately after injection, in this way more than 90 per cent. of the dose was recovered. Whereas, mice allowed to live for longer intervals show lower recoveries. Three hours after the injection a loss of 30 per cent. of the dose was observed, and a similar figure was obtained from experiments in which the animals were allowed to live 24 to 48 hours. The relationship of the percentage of the dose in the animal to that in the excreta and to the total recovery is shown in fig. 1.

Table III shows the percentage of the dose recovered by the time the concentration of sulphanilamide in the blood and in the urine was too small to estimate.

As the discrepancy in the volumes of distribution and the incomplete recovery of the drug from the urine could be explained by the drug

TABLE IV.—CONCENTRATION OF SULPHANILAMIDE IN RABBIT TISSUES.

Time, hours.			mg./100 gm. tissue.					Dose, mg./100 gm.
			Blood.	Liver.	Kidney.	Brain.	Muscle.	
3	Total .	.	2·53	3·00	5·37	1·54	2·16	5·71
	Free .	.	1·20	1·40	1·94	1·10	1·30	
3	Total .	.	4·36	5·28	10·08	3·00	5·08	5·00
	Free .	.	2·66	2·34	6·00	2·94	4·40	
3	Total .	.	3·24	3·88	8·29	1·50	2·93	4·65
	Free .	.	1·22	1·24	2·53	1·22	1·36	
6	Total .	.	1·22	1·42	4·42	0·82	0·90	5·33
	Free .	.	0·50	1·22	1·82	0·62	0·90	
6	Total .	.	1·20	1·42	2·24	0·70	0·90	4·25
	Free .	.·	0·44	0·82	0·86	0·70	0·74	
6	Total .	.	2·60	2·92	6·00	1·20	2·24	5·71
	Free .	.	0·90	1·70	2·64	1·10	1·10	
24	Total .	.	0·70	1·10	0·54	0·00	0·22	3·95
	Free .	.	0·00	0·80	0·00	0·00	0·00	
24	Total .	.	0·64	0·96	0·36	0·35	0·55	7·69
	Free .	.	0·52	0·42	0·55	0·24	0·47	
48	Total .	.	1·20	1·80	1·28	1·92	1·56	4·65
	Free .	.	0·76	1·10	0·76	0·50	0·30	
48	Total .	.	0·16	0·50	0·20	0·08	0·06	6·25
	Free .	.	0·00	0·50	0·00	0·00	0·00	
48	Total .	.	0·50	1·10	2·10	0·00	0·50	5·14
	Free .	.	0·00	0·90	1·24	0·00	0·00	
72	Total .	.	0·00	1·10	0·40	0·00	0·20	3·57
	Free .	.	0·00	0·84	0·00	0·00	0·10	
72	Total .	.	0·00	1·04	0·84	0·00	0·20	4·25
	Free .	.	0·00	0·96	0·40	0·00	0·00	
72	Total .	.	0·28	1·14	0·61	0·31	0·50	6·66
	Free .	.	0·18	0·78	0·43	0·24	0·37	

second hours would be occupied by absorption, and his observations at the fourth hour correspond to the first and second hours after intravenous injection.

It was also noticed that a part only of the dose given could be recovered from the urine. Fuller [1937], using rabbits, could only recover a fraction of the dose given from the urine.

The disparity in the calculated distribution volumes and the low recoveries of the drug from the urine, allows of two explanations: either the drug is not evenly distributed throughout the body water— that is, some is fixed by the tissues—or it is destroyed or changed into a form not estimated by diazotization.

The possibility of the compound being fixed by the tissues was then investigated, and it was found that some of the substance was fixed by the liver and kidney. However, the amount of drug which could be accounted for in this way was too small to supply the deficit between the dose and the quantity recovered from the urine. The destruction of the drug in the body was next studied, and it was found that, within three hours after the injection, a variable proportion of the compound had been destroyed or changed into a form not estimated by diazotization even after acid hydrolysis. Some evidence for the formation of oxidation products of sulphanilamide has been given by Rimington and Hemming [1939] and Shelswell and Williams [1940], so that a part at least of the fraction not accounted for in the experiments described may be converted into one of the compounds suggested by these authors. The loss of sulphanilamide all occurs in the first few hours while there is a high concentration in the body, it ceases when the concentration falls.

SUMMARY.

1. The concentration of sulphanilamide in the blood, one hour after intravenous injection, corresponds to an even distribution of the drug throughout the body water. The apparent volume of distribution, calculated six hours or more after the injection, becomes greater than the volume of the body.

2. A part only of the dose given can be recovered from the urine.

3. Estimations of the sulphanilamide content of rabbit's tissues, made at various intervals after intravenous injection, show higher concentrations in the liver and kidney than in the blood.

4. About 30 per cent. of the drug was destroyed in the body, so that it could not be detected by diazotization even after hydrolysis.

TABLE V.—CONCENTRATION OF TOTAL DRUG IN TISSUE/CONCENTRATION IN BLOOD.

Time, hours.	Liver/Blood.	Kidney/Blood.	Brain/Blood.	Muscle/Blood.
3	1·2	2·2	0·6	0·8
	1·2	2·3	0·7	1·2
	1·2	2·5	0·5	0·6
6	1·2	3·6	0·7	0·7
	1·2	1·9	0·6	0·7
	1·2	2·3	0·5	0·8
24	1·5	0·8	0·0	0·3
	1·5	0·6	0·5	0·9
48	1·5	1·1	1·6	1·3
	3·1	1·2	0·5	0·4
	2·2	4·2	0·0	1·0
72	∝	0·0	∝	∝
	∝	0·0	∝	∝
	2·2	1·1	2·0	4·0

TABLE VI.—THE PERCENTAGE OF DOSE RECOVERED FROM THE WHOLE ANIMAL AND EXCRETA.

Time, hours.	Number of observations.	Percentage recovered.		
		Body.	Excreta.	Total.
0	9	92·2 ± 0·75	0·0	92·2 ± 0·75
½	13	84·5 ± 4·5	3·2 ± 3·7	88·0 ± 3·7
1½	7	62·0 ± 1·4	17·6 ± 6·8	79·4 ± 5·4
3	8	31·6 ± 4·4	33·2 ± 3·5	65·0 ± 3·9
24	7	8·5 ± 1·1	62·4 ± 3·5	70·9 ± 4·0
48	5	6·7 ± 1·3	61·0 ± 4·6	67·7 ± 4·9

DISCUSSION.

The experiments show that while the volume of distribution of sulphanilamide calculated at the end of one hour after the injection may be explained on the hypothesis of an even distribution through the body water, the values obtained at longer intervals of time do not allow of this explanation. Marshall *et al.* [1937] found that, in the dog, sulphanilamide was evenly distributed through the body water. Since Marshall gave the drug orally and killed the dogs four hours later, the apparent contradiction may be explained; the first and

EFFECT OF VITAMIN B1 ON SURVIVAL TIME AFTER HÆMORRHAGE AND APPLICATION OF COLD. By W. D'A. MAYCOCK. From the Army Blood Supply Depot.

BLOTEVOGEL AND TONNUTTI [1939] stated that patients suffering from second and third degree burns of the extremities, and burns of the hand and face (severity not described), who were treated with vitamin B1 (10 mg.) and 10 per cent. glucose solution recovered more rapidly with less scarring than patients not so treated. Vitamin B1 and glucose were chosen as therapeutic agents because they increase the glycogen content of the liver. In burns the liver exhibits serous inflammation and a diminished glycogen level. Blotevogel and Tonnutti hoped in this way to maintain or increase the detoxicating property of the liver. They report, in addition to the apparently more rapid recovery of their patients, that pain was lessened after the injection of the vitamin.

Drury [1940], in a small number of rabbits, showed that vitamin B1 seemed to ameliorate their condition following the application of an injurious stimulus, to be described later. The experiments performed by Drury have been repeated with slight modifications, to ascertain whether vitamin B1 would be of value in combating the effects of hæmorrhage and cold in rabbits. During the course of these experiments Govier and Greer [1941] have produced evidence that vitamin B1 prolongs the life of dogs subjected to hæmorrhage, prevents or lessens hepatic glycogenolysis and decreases the amount of keto-acids observed in the blood after various types of shock. The reason for a relative deficiency of the vitamin in the condition studied by Govier and Greer (shock following hæmorrhage) is not clear; they suggest that bleeding may in some way decrease the available amount of vitamin B1 in the tissues; but they also emphasize that the increases observed in blood sugar and keto-acids may be quite unrelated to the "shock syndrome" itself.

METHODS.

Rabbits weighing between 1 and 3 kg. were used. They were of no particular breed and were fed before operation on a mixed diet of bran and fresh vegetables. More recently bran has been omitted from the diet because of the difficulty in obtaining it.

29

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RESULTS.

The results in the first series of the experiments are shown in Table I.

Of 10 untreated animals 3 survived: death rate 70 per cent.

„ „ ‎ treated „ 5 „ : „. „ 50 „

The difference between these small groups is not significant. It was noticed that the treated animals seemed to make a more rapid clinical recovery. The effects of the anæsthesia passed off more quickly and the treated animals sat up or moved about in their cage 1–2 hr. before the untreated animals were able to raise themselves from the prone position. This observation was made even in experiments Nos. 30 and 31, in which, although treated, the animals died within 12 hr.

It seemed therefore that vitamin B1 might be exerting only a temporarily beneficial effect upon the animals' condition and that the criterion of successful treatment adopted, survival of the animal, was too severe. A second series of experiments was therefore performed in which transfusions were given 3 hr. after completion of the operation, when the beneficial effect of vitamin B1 was still apparent. If vitamin B1 were responsible for the clinical improvement it was to be expected that the number of survivals in the animals receiving vitamin B1 and transfusions would be significantly greater than the number of survivals in animals receiving only transfusions.

Preliminary experiments were performed to find the amount of blood which could be transfused into rabbits without lowering the mortality rate too greatly. It was decided to transfuse half the volume of blood withdrawn.

Accordingly a transfusion equivalent to half the volume of blood previously removed was given to each of 20 rabbits, 10 of which had received intravenous injections of vitamin B1 after completion of the operation.

The results obtained in the series of experiments are shown in Table II. Of 10 rabbits which received transfusions but no vitamin B1 6 died: death rate 60 per cent. Of 10 animals which received both transfusions and injections of vitamin B1 5 died: death rate 50 per cent.

The difference between these small groups is not significant. The slight improvement in the survival rate of animals receiving transfusions but no vitamin compared with that of the animals which were not transfused and received no vitamin is not significant and is of the same order as that seen when groups of animals receiving vitamin are compared with those not receiving vitamin in both series.

An examination of the survival period of the animals in each group which died did not reveal any significant prolongation of life in those treated with vitamin B1.

Vitamin B1 was not given in doses directly proportional to the body

Anæsthesia was produced in earlier experiments by the intra-peritoneal injection of Nembutal (Abbot) 35 mg./kg. In later experiments chloroform was used to induce anæsthesia, followed by ether.

After shaving the abdomen and groin, the right femoral artery was exposed aseptically, and cannulated. Blood equivalent to 1·6 per cent. of the body weight was rapidly removed. The abdomen was then opened aseptically. The small intestines and cæcum were withdrawn and placed in a small rubber bag around which small cubes of ice were packed. A rubber bag was used to prevent the possible absorption of fluid in the peritoneal area. The cooling process was continued until the rectal temperature had fallen to 32° C., after which the intestines were replaced in the peritoneal cavity and the abdominal and inguinal incisions closed with continuous silk sutures. The rabbit was returned to its cage in the animal room, in which the temperature ranged between 16° C. and 21° C. The animals began to recover from the anæsthetic 1–2 hr. after the completion of the operation. Those which eventually died usually remained huddled in the cage, the legs splayed apart and the head resting on the floor; they made no effort to eat. Some animals, which subsequently died within 24 hr., made a more complete recovery, sitting up normally and moving about. After some hours they relapsed and lay in the prone position until death. The animals which survived were usually sitting up 5–6 hr. after they had recovered from the anæsthetic and began to eat on the day following the operation, and in 3 or 4 days were clinically normal. All surviving animals were killed on the 6th day after operation.

It was noticed that the rectal temperature fell rapidly to as low as 28° C. after the intestines had been replaced in the peritoneal cavity, and might remain low for some hours after the animal was sitting up.

Hæmoglobin and hæmatocrit determinations on samples of blood (2 c.c.) drawn from the femoral artery were made during the experiment. Post-operatively the hæmoglobin level was determined in blood obtained by pricking the marginal vein of the ear.

In some experiments the blood pressure was measured in the central artery of the intact ear with a Grant's capsule. The ear was warmed before each reading until the vessels appeared to be fully dilated.

In the treated animals, vitamin B1 (Benerva) was injected intravenously $\frac{1}{2}$–1 hr. after completing the operation, in doses ranging from 2–5 mg. (the solution contained 5 mg. per c.c.). The volume of fluid injected was so small that it was considered unnecessary to inject a similar volume of fluid without vitamin B1 into the untreated animals. In some animals a transfusion of blood equal to half that removed was given 2–3 hr. after the closure of the abdomen. It was not possible to make any observation on the biochemical changes in the blood.

TABLE II.

No.	Wt., kg.	Blood loss, per cent. body wt.	Rectal Temp. °C. Initial.	Rectal Temp. °C. Final.	Exposure of intestines, min.	Hæmatocrit. Initial.	Hæmatocrit. End of hæm.	Hæmatocrit. End of exper.	Vit. B1, mg.	Result.
48	2·3	1·52	37·0	32·0	43	43·5	39·0	27·5	..	Died in 48 hr.
49	2·10	1·62	36·0	32·0	29	36·0	33·5	32·0	..	Died in 48 hr.
50	1·48	1·61	37·6	32·0	62	36·5	30·0	28·0	..	Died in 12 hr.
55	2·16	1·66	36·3	32·0	23	38·0	35·0	35·0	..	Died in 12 hr.
63	1·59	1·63	36·5	32·0	29	..	..	..	..	Died in 72 hr.
67	1·59	1·63	30·2	32·0	14	33·0	32·0	30·5	..	Died in 24 hr.
53	1·70	1·59	36·5	32·0	18	41·0	40·0	..	..	Survived.
59	2·04	1·62	39·0	32·0	49	39·0	39·0	..	..	Survived.
61	2·73	1·65	37·2	32·0	50	..	..	32·0	..	Survived.
65	1·53	1·63	37·0	32·0	28	36·0	33·0	32·0	..	Survived.
51	2·27	1·50	34·0	32·0	11	38·0	..	32·0	3·75	Survived.
56	2·21	1·61	35·7	32·0	36	40·0	35·0	33·0	5·0	Survived.
58	1·82	1·64	37·3	32·0	30	38·0	40·0	35·0	5·0	Survived.
60	2·04	1·62	37·3	32·0	48	41·0	40·0	37·0	5·0	Survived.
62	2·73	1·68	37·0	32·0	40	38·5	37·0	32·0	5·0	Lived for 5 days.
52	2·17	1·66	38·0	32·0	35	37·0	42·0	38·0	5·0	Died in 24 hr.
57	1·59	1·63	36·8	32·0	28	41·0	37·0	37·5	5·0	Died in 24 hr.
64	1·02	1·55	35·3	32·0	15	31·0	31·0	28·0	5·0	Died in 24 hr.

· TABLE I.

No.	Wt. kg.	Blood loss per cent body wt.	Rectal temp. °C		Exposure of intestines, min.	Hæmoglobin (Sahli) per cent.							B.P. (oar) mm. of mercury.					Vit. B1, mg.	Result.
			Init.	Final.		Init.	End of exper.	24 hr.	48 hr.	72 hr.	96 hr.	120 hr.	Init.	End of exper.	24 hr.	48 hr.	72 hr.		
15	2·18	1·60	39·0	32·0	58	66	69	..	..	..	..	..	75	55	40	..	..	..	Died in 55 hr.
16	1·59	1·57	38·0	32·0	45	66	..	..	..	..	..	..	75	20	..	..	..	..	Died in 24 hr.
21	2·28	1·70	38·0	32·0	92	91	85	84	65	..	..	..	70	45	70	55	..	..	Died in 2½ days.*
29	2·71	1·66	37·0	32·0	70	..	..	..	..	..	..	..	80	55	..	..	..	..	Died in 8 hr.
32	2·16	1·62	36·0	32·0	28	57	40	..	..	..	..	..	70	35	..	..	..	..	Died in 36 hr.
41	1·9	1·57	36·8	32·0	31	68	58	..	..	..	..	..	80	35	..	..	..	..	Died in 24 hr.
43	1·48	1·61	35·0	32·0	17	62	55	..	..	..	..	..	105	55	..	..	..	..	Died in 8 hr.
25	3·15	1·57	36·5	32·0	56	45	38	34	32	30	32	38	70	45	40	40	45	..	Survived.
35	2·26	1·63	39·0	32·0	45	54	38	42	40	41	..	..	80	30	..	..	..	..	Survived.
38	2·23	1·61	36·0	32·0	28	62	53	42	41	45	44	58	70	50	45	50	75	..	Survived.
19	1·05	1·61	39·0	32·0	25	49	37	..	..	..	..	..	70	35	..	..	..	2·0	Survived.
22	3·0	1·50	38·0	32·0	84	72	60	60	40	40	42	47	90	55	80	..	75	2·0	Survived.
24	2·48	1·45	36·0	32·0	74	95	70	51	47	33	42	..	85	60	85	60	75	3·0	Survived.
33	2·51	1·67	36·0	32·0	19	53	44	..	56	52	52	..	80	40	..	..	..	4·5	Survived.
40	2·25	1·55	36·0	32·0	27	71	58	52	56	..	55	65	80	70	65	70	75	3·75	Survived.
30	2·05	1·65	38·0	32·0	85	57	49	..	..	..	..	..	55	30	..	..	..	3·0	Died in 12 hr.
31	2·0	1·60	38·8	32·0	69	85	52	..	..	..	..	..	110	40	..	..	..	4·5	Died in 12 hr.
36	2·26	1·50	37·0	32·0	40	55	40	55	..	..	..	..	75	..	..	..	..	4·5	Died in 48 hr.
42	1·28	1·64	37·8	32·0	14	70	43	..	..	..	..	..	60	25	..	..	..	4·5	Died in 24 hr.
45	1·7	1·60	37·0	32·0	35	75	60	..	..	..	..	..	130	40	..	..	..	4·5	Died in 14 hr.

* Hæmoconcentration observed during operation: lowest value for hæmoglobin 61 per cent.

OBSERVATIONS ON THE EFFECTS OF RENAL ISCHÆMIA UPON ARTERIAL PRESSURE AND URINE FLOW IN THE DOG. By E. B. VERNEY and MARTHE VOGT.[1] From the Pharmacology Laboratory, Cambridge.

THE experiments to be described in this paper were planned in order to follow closely, in the dog, the changes produced by compression of the renal artery in the arterial blood pressure and in the secretion of urine during water diuresis; and they have been extended with a view to discovering whether or no the liver plays a paramount rôle in the inactivation of the pressor substance liberated by ischæmic renal tissue.

METHODS.

Fully grown bitches were used, and they were prepared for blood-pressure measurement by the method of the carotid loop with denervated sinus [see Verney and Vogt, 1938 *b*]. When the animal had been trained to lie quietly on a warmed table, and the arterial pressure readings were constant from day to day, a compression unit was implanted around a renal artery (usually the right) at its origin from the aorta.

Three types of compression unit have been used.[2] That most frequently employed has been described by Rydin and Verney [1938; see fig. 17 in their paper]. The fine pressure-tube extension was, however, covered with a weave of fine silk (diam. 150 μ) over which a thin coating of latex was then deposited: injection of liquid into the tubing thus caused quantitative expansion of the rubber membrane, which later lay in apposition with the renal artery. Each unit was roughly calibrated before use by selecting a piece of rubber tubing of a size to fit snugly into the arterial channel of the bakelite block, and perfusing it with water at pressures varying between 150 and 200 mm. Hg. The volumes of injected liquid needed definitely to arrest and to slow the perfusion flow were then determined. They were of the order

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[1] Alfred Yarrow Research Fellow of Girton College while this work was being done.

[2] These units have been made for us by Messrs. C. F. Palmer (London) Ltd. We wish to record our gratitude to Mr. E. W. H. Ellis for the care he has taken in the detail of their construction.

weight, but, since the· heavier rabbits did not die, and the lighter ones survived, it would not seem that any different result would have been obtained had the doses been chosen according to weight.

In Table I the rabbits which died had an average weight of 1·86 kg. and received 2·26 mg./kg. vitamin. while those that survived had an average weight of 2·26 kg. and received 1·35 mg./kg. vitamin. In Table II the corresponding values are 1·57 kg. and 3·10 mg./kg.; 2·21 kg. and 2·02 mg./kg. vitamin.

It will be noticed that the time of exposure of the intestines to cold necessary to lower the rectal temperature to 32° C. varies considerably from animal to animal in each group, but no obvious relationship between this period of time and the survival or death of the animals is apparent.

Hæmoconcentration was observed in only 4 experiments (Nos. 15 and 21 in the series of untreated animals in Table I, and Nos. 52 and 58 in the series of treated animals in Table II). This is in· contrast with the experiments reported by Drury in which hæmoconcentration amounting to 10–20 per cent. was observed after the operation. In fact the rapidity with which hæmodilution occurred after bleeding is striking in almost all the animals observed.

SUMMARY.

Under the experimental conditions described it has not been possible to demonstrate that vitamin B1 exerts any significant beneficial effect upon the survival rates of rabbits subjected to bleeding and the application of cold to the intestines.

Transfusion, equivalent to half the blood removed, 3 hr. after hæmorrhage, has no significant effect upon the survival rate.

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compression is released, depends upon the elasticity of the rubber tube F, and this return may be assisted by applying a slight negative pressure by means of a syringe attached to the end of· the extension tube. We shall refer to this unit as Type II. Two modifications of this have occasionally been used when the production of prolonged partial obstruction of the renal artery has been desired. In the one, the rubber tube F (fig. 1), the silver cylinder J, and the main block B are each 2 mm. longer, and the piston surface adjacent.to the artery is scooped, so that when the piston is at the end of its thrust the lumen of the artery is only partially reduced. In the other, the extension tube consists in a fine closely wound nickel steel spiral, 1 mm. in internal diameter, one end of which is soldered to the boss K (fig. 1), the other to a 2-cm. length of fine silver tubing; the spiral is covered by a thin rubber tube, and this in turn by a weave of fine'silk and a thin coating of latex. The silver piston is bored centrally, and the bore is counter-sunk at its opening in the scooped surface of the piston. Through the bore is passed a thread of No. 4 silk knotted at the end, and the knot is pulled into the countersink and covered in with silver solder. The free end of the silk is then threaded through the block E and the extension tube. The rubber tube F is of such a length that the piston is thrust firmly to the extreme of its travel, and the piston is brought back and held in the position shown in fig. 1 by pulling the silk thread, and fixing it to the end of the extension tube. When the unit has been implanted, partial obstruction of the renal artery is effected by releasing the thread, and decompression of the artery by tightening it again.

A third type of unit is illustrated in fig. 2. The extension tube has the same construction as that in the Type II modification which has just been described. The unit is made of silver, and partial obstruction of the renal artery is effected by the rotation of a cam (D, fig. 2). The cam is operated by two threads which are fixed at F, F', and lie in the slot H; their free ends are threaded through the boss G and the extension tube. Traction on the thread attached at F rotates the cam through 90° from the position shown in the figure, and so diminishes the size of the arterial channel A: the cam is brought back to its original position by traction on the thread attached at F'. The surface of the cam is so shaped that it rides smoothly across the arterial wall as this is being compressed. Ascending infection, after implantation of the unit, is prevented by injecting along the extension tube a little warmed phenol ointment at the time of the operation. We shall refer to this unit as Type III.

The unit selected was implanted by the technique of Rydin and Verney [1938]. The extension tube from the unit was conducted to the exterior through either uterine horn, that route being chosen which ensured the minimum displacement of the renal artery from its natural position. When the contra-lateral horn was selected, this was

of 100 and 50 c.mm. respectively. This type of unit has been used in the majority of experiments; but it should be recognised that although such units are mechanically satisfactory for occlusion of the renal artery for short periods, they are less so for partial obstruction for long periods: liquid injected up the extension tubing in a volume sufficient to produce partial obstruction is, as the observations recorded later show, incompletely recoverable when it is released after periods varying from half an hour to seven hours. The degree of obstruction, therefore, is probably diminishing during these periods. When the effects of prolonged obstruction of the renal artery were to be followed, the extension tubing was clamped after the requisite volume of liquid
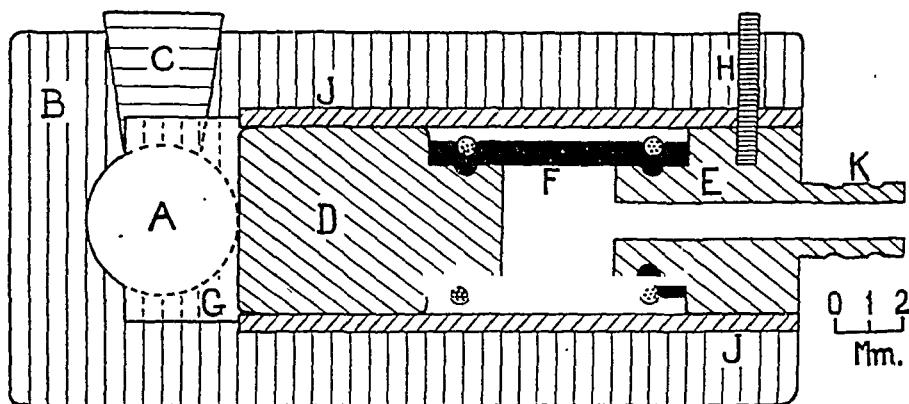
Fig. 1.—Compression unit, Type II. A: channel for renal artery. B: bakelite block 21 × 10 × 10 mm. C: bakelite wedge. D: silver piston. E: silver block. F: rubber tube. G: cylindrical channel into which the piston slides to compress the artery. H: silver plate securing E within the silver cylinder J. K: boss to which extension tube is attached.

had been injected, and the end of the tubing was closed by inserting into it, and fixing by ligature, a short silver wire. The clamp was then removed, and the end of the tubing tucked away in the vaginal fornix. We shall refer to this unit as Type I.

A second type, which we have sometimes used, is illustrated in fig. 1. The extension tube is of the same construction as in Type I, and this tube, together with the expansion chamber of the unit, is similarly filled with liquid when the unit is assembled. Liquid injected through the extension tubing causes the piston D (fig. 1) to move in an anterior direction and to compress the renal artery lying in the channel A. As in Type I, the volumes of liquid necessary to cause complete occlusion of, and partial obstruction in a tube lying in the arterial channel A, were determined before the unit was implanted; they were of the order of 110 and 50 c.mm. respectively. In this type of unit, the return of the piston to the position shown in fig. 1 when the

operations were performed under ether anæsthesia with full surgical precautions.

No. 183. 4.3.38: denervated carotid loop made. 22.4.38: unit—Type I—implanted at origin of right renal artery; left kidney removed; fundus of bladder excised [see Rydin and Verney, 1938], and self-retaining catheter left in bladder-stump. 7.5.38: signs of intermittent ureteric obstruction appeared. 10.5.38: laparotomy; ureter freed, and unit replaced by another of the same type; uninterrupted recovery.

No. 196. 13.5.38: denervated carotid loop made. 8.6.38: unit—Type I—implanted at origin of right renal artery; extension tube passed dorsal to I.V.C. and aorta, and into left uterine horn; left kidney excised. 16.6.38: splanchnic nerves on each side divided as they entered the abdomen.

No. 208. 25.11.38: denervated carotid loop made. 20.1.39: unit—Type I—implanted at origin of right renal artery, and held adjacent to aorta by ligature passing around aorta between origins of renal arteries; extension tube passed dorsal to I.V.C. and aorta, and into left uterine horn; all visible nerve filaments on right renal artery and vein divided, and stripped from the vessels; right splanchnic nerves exposed by dividing peritoneum attaching upper pole of kidney to liver; bilateral section of splanchnic nerves. 27.1.39: left kidney removed through lumbar route. 15.3.39: right kidney lifted from its bed by dividing between ligatures all its attachments other than the artery, vein and ureter; all ancillary vessels were by these means divided, along with all nerve filaments; kidney replaced in its natural position by tying together each pair of ligatures between which the perirenal tissue had been divided.

No. 219. 17.2.39: denervated carotid loop made. 25.4.41: unit—Type I—implanted at origin of right renal artery, and held adjacent to aorta by ligature passing around aorta between origins of renal arteries; extension tube passed dorsal to I.V.C. and aorta, and into left uterine horn; all visible nerve filaments on right renal artery and vein divided, and stripped from the vessels; right splanchnic nerves exposed by dividing peritoneum attaching upper pole of kidney to liver; bilateral section of splanchnic nerves. 2.5.41: left kidney removed through lumbar route.

No. 181. 28.1.38: denervated carotid loop made. 16.2.38: unit—Type I—implanted at origin of left renal artery; extension tube passed between aorta and I.V.C., and into the right uterine horn; fundus of bladder excised, and self-retaining catheter left in bladder-stump; right kidney removed.

No. 206. 21.10.38: denervated carotid loop made. 16.11.38: unit—Type I—implanted at origin of right renal artery; extension tube passed dorsal to I.V.C. and aorta, and into left uterine horn; right kidney denervated; bilateral section of splanchnic nerves; left kidney removed.

No. 238. 18.7.39: unit—Type II—implanted at origin of right renal artery, and held adjacent to aorta by ligature passing around aorta just posterior to origin of left renal artery; extension tube passed dorsal to I.V.C. and aorta, and into left uterine horn; right kidney denervated; bilateral section of splanchnic nerves.

No. 257. 13.1.40: unit—Type III—implanted at origin of right renal artery, and held adjacent to aorta by ligature passing around aorta just posterior to origin of left renal artery; extension tube passed dorsal to I.V.C. and aorta, and into left uterine horn; right kidney denervated; bilateral section of splanchnic nerves.

No. 258. 25.1.40: unit—Type III—implanted at origin of right renal artery; upper end of extension tube held against aorta by two ligatures passing

usually reached by passing the tubing dorsal to both inferior vena·cava and aorta.   The implantation of the unit always involved the section of a proportion of the renal nerves; and mention will be made in each instance in which complete denervation of the kidney or section of the
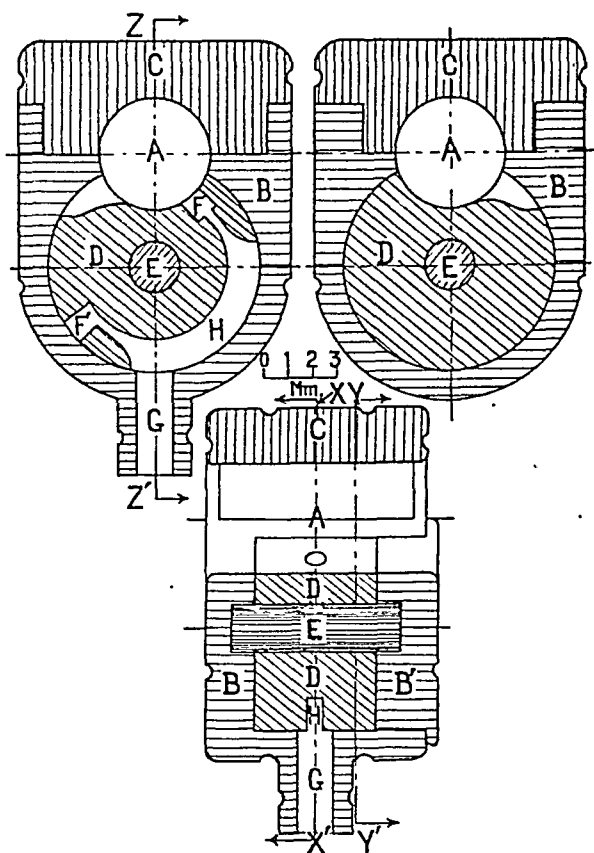
Fig. 2.—Compression unit, Type III.   Below, sectional plan; above (left), section through XX' and (right) section through YY', looking in direction of arrows.   The sectional plan is a section through ZZ' looking in the direction of the arrows.   A: arterial channel.   B: silver block.   B': silver side cap. C: silver cap.   D: silver cam.   E: silver spindle.   F, F': countersinks for knotted ends of threads passing to the exterior through the slot H, the boss G, and the extension tube.

splanchnic nerves was carried out in addition.   As a rule, the kidney on the side opposite to that on which the unit had been implanted was excised either at the time of this operation or, and then by the lumbar route, some days later.

We propose now to give a short description of the operative procedure in each of the animals before and during the periods in which the effects of obstruction of the renal artery were observed.   All the

urine flow showing close temporal accord. Although a little fat and albumen were detected in the first few samples of urine after release of the artery, the amount of albumen varying directly with the period of preceding occlusion, they rapidly disappeared in subsequent samples; and in the experiment where occlusion of the renal artery was maintained for 10 minutes, a trace only of albumen was present 40 minutes
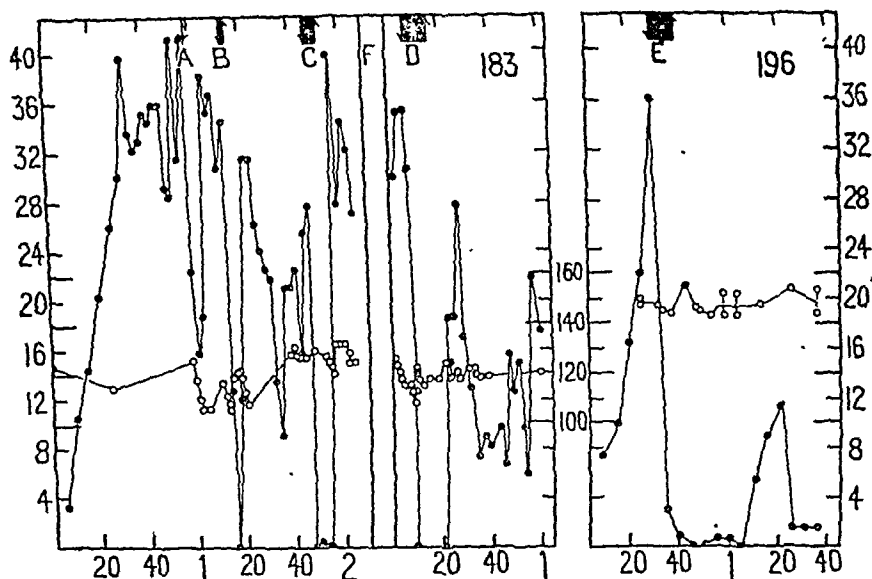
Fig. 3.—Left: the effects of occlusion of the right renal artery for 30 sec. (A), 120 sec. (B), and for 300 sec. (C), in dog 183, on 30.4.38; 200 c.c. of water by stomach tube 170 minutes before, and 250 c.c. 16 minutes before zero time. F: interval of two days. At D the artery was occluded for 600 sec. on 2.5.38; 250 c.c. of water by stomach tube 6¼ hours before, and 300 c.c. 130 minutes before zero time. Right: the effects of occlusion of the right renal artery for 600 sec. (E), in dog 196, on 22.6.38; 270 c.c. of water by stomach tube 175 minutes before, and 260 c.c. 15 minutes before zero time. Closed circles: urine flow. Open circles: systolic blood pressure. Ordinates: right and left, urine flow in c.c./15 min.; intermediate, blood pressure in mm. Hg. Abscissæ: time in minutes and hours.

after the release of the artery. On no occasion was hæmoglobin detected in the urine. As will be seen from the figure, occlusions of the renal artery for periods from 30 to 600 sec. in this animal were neither accompanied nor followed by any appreciable change in arterial pressure. In another animal (No. 196), in which a similar operation had been performed, occlusion of the renal artery for 10 *minutes*, fourteen days after implantation of the unit, was accompanied and followed by suppression of urine for a total period of 36 minutes, without there being an appreciable change in arterial pressure (fig. 3, right-hand side). The persistence of the suppression of urine for 26 minutes after the release of the occluding pressure, may be connected

around aorta posterior to left renal artery; extension tube passed into right uterine horn, the upper part of which is excised; right kidney denervated; bilateral section of splanchnic nerves. 30.1.40: operation for reduction of intussusception; recovery.

When the animal had fully recovered from the operation of im-. plantation of the compression unit, it was given 250 or 300 c.c. water by stomach tube, a self-retaining catheter was introduced into the bladder, the dog was laid in a comfortable position on a warmed table and, after the external end of the extension tube had been suitably prepared for subsequent compression of the renal artery, blood-pressure readings were taken repeatedly from the carotid loop. When a satis-factory base line of arterial pressure had been obtained and diuresis was at its height, the renal artery was completely or partially obstructed for varying periods, and the effects of these procedures on arterial pressure and diuresis were followed.

On a few occasions, the effects of compression of the renal artery on the arterial blood pressure were directly recorded by means of a cannula introduced, under local anæsthesia and with aseptic precautions, into a branch of the femoral artery, the technique being the same as that described in a previous paper [Verney and Vogt, 1938 b].

In a few dogs, the compression of the renal artery was accompanied by excitement, and the blood pressure rose immediately. Such animals were, of course, useless for our purpose, but bilateral section of the splanchnic nerves usually abolished these sensory reactions. With the others the smoothest curves of blood-pressure response were obtained in those experiments in which the animal fell asleep, and was apparently unconscious of the manipulations.

Operative procedures on animals other than those to which we have just referred, will be described in their appropriate contexts.

RESULTS.

The Effects of Short but Complete Obstruction of the Renal Artery.

We propose, first, to describe the effects of complete occlusion of the renal artery for periods ranging between 2 and 300 (rarely 600) seconds, taking for this purpose the results obtained in five animals: Nos. 183, 196, 208, 219, and 181. In fig. 3 (left-hand side) are shown the effects of arterial occlusions for 30, 120, and 300 sec. eight days, and for 600 sec. ten days after the implantation of a compression unit in dog 183. The one half-minute occlusion caused no suppression of the flow of urine, the two-minute occlusion caused suppression for 3 minutes, the five-minute for 8 minutes, and the ten-minute occlusion caused suppres-sion for 13 minutes. The recovery of urine flow was in each instance rapid and complete. These results conform with those described by Rydin and Verney [1938], the arterial occlusion and suppression of
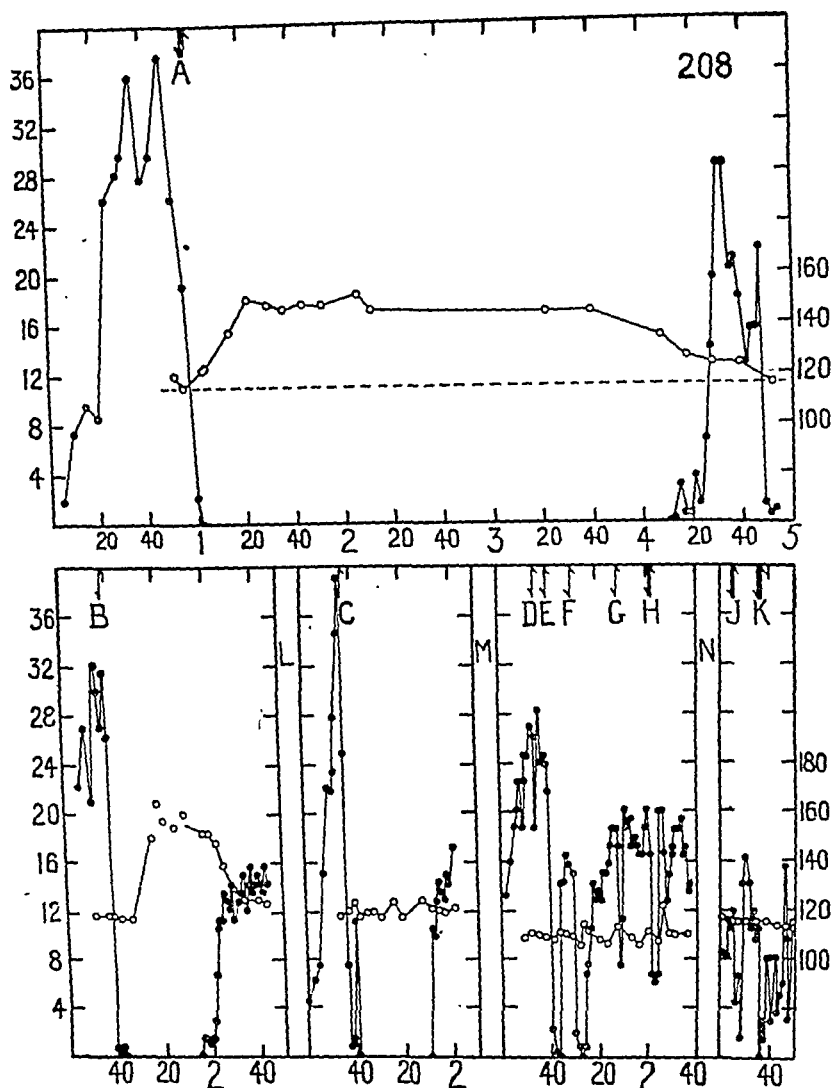
Fig. 4.—Dog 208. A: occlusion of the right renal artery for 120 sec. on 10.2.39; 300 c.c. of water by stomach tube 2 hours before, and again 3 minutes before zero time. B: arterial occlusion for 30 sec. on 13.2.39; 200 c.c. of water by stomach tube 5 minutes before zero time. C: arterial occlusion for 2 sec. on 15.2.39; 300 c.c. of water by stomach tube 150 minutes before, and again 15 minutes before zero time. D, E, F, G, and H: arterial occlusions for 10, 10, 10, 30, and 120 sec. respectively on 2.3.39 during water diuresis. J and K: arterial occlusion for 120 and 180 sec. respectively on 18.3.39; 130 c.c. of water by stomach tube 110 minutes before, and 280 c.c. 50 minutes before zero time. L: interval of two days. M: interval of fifteen days. N: interval of sixteen days. Closed circles: urine flow. Open circles: systolic blood pressure. Ordinates: left, urine flow in c.c./15 min.; right, blood pressure in mm. Hg. Abscissae: time in minutes and hours.

with the fact that the resting blood pressure was raised, or that the splanchnic nerves had been divided six days earlier. When the flow of urine returned, the first sample contained fat, hæmoglobin, and a large amount of albumen, and a few granular casts were seen in the centrifuged deposit. Subsequent samples contained fat, hæmoglobin, and albumen in rapidly diminishing amounts. The experiments on these two animals show that occlusion of the renal artery for periods up to 10 minutes may lead to no appreciable change in the arterial blood pressure, even when the flow of urine is suppressed for as long as 36 minutes, and when the damage to renal function is so severe as to lead to the temporary appearance of casts, blood, and albumen.

Quite different responses to short periods of arterial occlusion were, however, obtained in three other animals (Nos. 208, 219, and 181). In No. 208 the splanchnic nerves were divided, and the right renal vessels stripped of all visible nerve filaments when the unit was implanted. *Twelve* days later, occlusion of the renal artery for 120 sec. was followed by suppression of urine for more than 3 hours; and when a catheter specimen of urine was taken 18 hours after the occlusion, it was found to contain fat, albumen, and red blood corpuscles. The arterial blood pressure, which before occlusion was between 100 and 110 mm. Hg, had risen to 120 mm. 5 minutes later, and gradually rose to reach 140 mm. 40 minutes after the occlusion. The pressures 2 hours, 4 hours, and 6¾ hours after the occlusion were 146, 137, and 118 mm. respectively. *Eight* days later the experiment was repeated, and the results are shown in fig. 4 (above). As is seen, the arterial occlusion was followed by suppression of urine for 3¼ hours. The rapid recovery of urine flow was accompanied by the appearance in the urine of fat and albumen and red blood corpuscles, and the albumen had almost vanished 35 minutes after the flow of urine began to return. During the period of suppression of urine, the blood pressure was definitely raised, and its course is plotted in the figure. *Three* days later, the effects of occlusion of the renal artery for 30 sec. were determined, and the results are shown in fig. 4 (below; left). The blood-pressure and urine-flow responses were similar to those of arterial occlusion for 120 sec., but of shorter duration; and it was found that occlusion for so short a period as 2 sec., although leaving the course of blood pressure unaffected, suppressed the urine flow for as long as one hour (fig. 4, C). *Fifteen* days after the experiment illustrated by fig. 4 C, the effects of occluding the renal artery for 10, 30, and 120 sec. were again determined. They are shown in fig. 4 at D, E, and F, G, and H; the prolonged after effects which were observed earlier have now completely disappeared, the urine flow being merely transitorily inhibited, and the blood pressure not being appreciably affected. The results have thus become similar to those obtained in dogs 183 and 196 and illustrated in fig. 3. They were the same when the experiment was repeated the following day, and again

urine observed in these animals are due to some change within the kidney elicited by the short periods of arterial occlusion, and persisting long after the occluding pressure has been withdrawn. The view that this change is a long lasting vasoconstriction, is supported by the finding that the responses resemble those to prolonged but partial obstruction of the renal artery, produced at a time when the prolonged
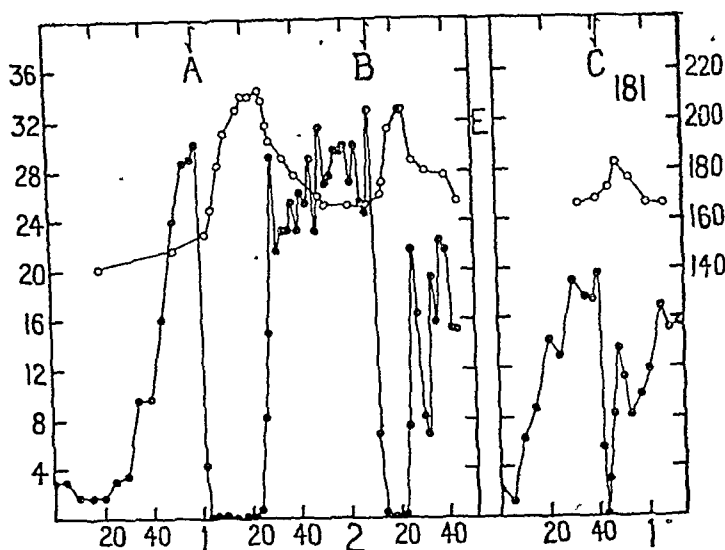
Fig. 5.—Dog 181. A and B: occlusions of the left renal artery for 30 sec. and 15 sec. respectively on 28.2.38; 250 c.c. of water by stomach tube 3 hours before, and 300 c.c. 22 minutes before zero time. E: interval of four days. C: arterial occlusion for 5 sec.; 250 c.c. of water by stomach tube 4 hours before, and again 2 minutes before zero time. Closed circles: urine flow. Open circles: systolic blood pressure. Ordinates and abscissæ as in fig. 4, q.v.

effects of short periods of occlusion have disappeared (see fig. 6). We have not enough information to sanction inference of the conditions upon which these prolonged effects of short periods of arterial occlusion depend. It may be that as a result of division of the sympathetic supply (No. 208), or impairment of the blood supply (No. 181), or both (No. 219), nerve elements in the hilum of the kidney become supersensitive in the sense that short periods of asphyxia lead to long lasting constriction of the small intrarenal vessels, a supersensitivity which later disappears. Experiments have not been made earlier than nine days after the unit has been implanted; and both the prolonged suppression of urine and the prolonged rise in blood pressure have been observed when the renal artery has been occluded for short periods (5 to 120 sec.) between nine and twenty-three days after the operation. Although there is usually temporal accord between the blood-pressure and urine-flow responses, the two effects are dissociable (see fig. 4, C): the former disappeared before the latter in animal 208, in which the

sixteen days later (fig. 4, J and K), on this last occasion three days after ligation of all vessels ancillary to the renal artery and vein, and division of any nerve fibres which had escaped section when the unit was implanted or had regenerated since.

An attempt was made to repeat this experiment as closely as possible in another animal, No. 219. Thirteen days after implantation of the unit—the operation, as in No. 208, was accompanied by bilateral division of the splanchnic nerves, and by the stripping of the right renal vessels of all visible nerve filaments—the arterial pressure was 170 mm. Hg; the unit was a little too small for the artery, and the high resting pressure was an expression of this. The renal artery was occluded for 120 sec., and the arterial pressure which, during the 30 minutes before the occlusion, varied between 170 and 174 mm. Hg, climbed to 195 during the succeeding 10 minutes, and remained between 188 and 194 for a subsequent period of 2 hours 10 minutes. It then gradually fell to reach a value of 168 mm. Hg two hours later. The rate of urine flow, 5 c.c./15 min. before the occlusion, varied between 0·1 and 0·7 c.c./15 min. during the period of raised blood pressure, and then climbed to 3·4 c.c./15 min. during the period of recovering blood pressure. Although the resting blood pressure of this animal was raised, and the urine contained macroscopic blood throughout the experiment just described, the prolonged and parallel effects on blood pressure and urine flow of this short period of occlusion resemble those observed in animal 208.

The remaining animal (No. 181) in which the effects of short complete occlusion of the renal artery were determined, also reacted by prolonged suppression of urine accompanied by raised arterial pressure. When the unit was being implanted, no attempt to denervate the kidney was made, and the splanchnic nerves were not divided. The blood pressure of this animal before operation was 120 mm. Hg. Three days after operation it had risen to between 140 and 150 mm., and never returned to the normal level. The raised pressure was presumably due to the fact, as disclosed at the post-mortem examination, that the position of the unit was such that the renal artery was kinked between its origin and its entry into the unit, the vessel thereby being partially obstructed at this site. Nine days after the operation, the effects of occlusion of the renal artery for 30 sec. were determined on two occasions. Suppressions of urine flow for 17· and 21 minutes, accompanied by rises in pressure from 152 to 170 mm. Hg and from 147 to 197 mm. Hg respectively, were encountered. Fig. 5 illustrates the urine-flow and blood-pressure responses of this animal to arterial occlusion for 30, 15, and 5 sec. The results shown in the left part of the figure were obtained twelve days, those in the right part sixteen days after the implantation, and the responses are seen to be directly related in degree to the period of arterial occlusion. The resting pressure of this animal was gradually rising, and the suppression of urine flow by short periods of arterial occlusion disappeared, although their effects on the arterial blood pressure persisted.

Evidently the prolonged rise in blood pressure and suppression of

first detectable about 10 minutes after the renal artery is first compressed, reaching a value of about 30 mm. Hg during the period of obstruction, and vanishing about 50 minutes after the compression is released.

In two other animals (206 and 183), the effects of partial obstruction of the renal artery for periods longer than in dog 208 were observed:
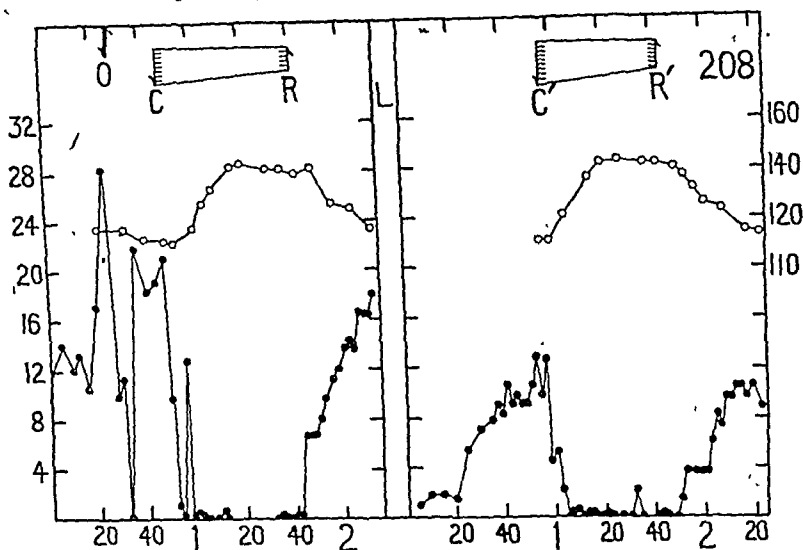
FIG. 6.—Dog 208. O: occlusion of right renal artery for 120 sec. on 13.3.39. At C the renal artery was partially obstructed, and at R the obstruction was released. 200 c.c. of water were given by stomach tube 190 minutes before, and 300 c.c. 15 minutes before zero time. L: interval of 7 days. At C' the renal artery was partially obstructed on 20.3.39, and at R' the obstruction was released. 250 c.c. of water were given by stomach tube 150 minutes before, and 300 c.c. 20 minutes before zero time. The scales above C, C' and R, R' represent the volumes of liquid injected into and recovered from the unit during compression and decompression of the renal artery: each division represents 10 c.mm. Closed circles: urine flow. Open circles: systolic blood pressure. Ordinates and abscissæ as in fig. 4, q.v.

they, too, are recorded in Table I. The periods varied between 2 and 7 hours, and it is probable that the degree of obstruction was somewhat less than in dog 208, seeing that the latent period of the onset of the rise in pressure was usually a little longer, and the degree of rise usually a little less. Moreover, the urine flow was usually inhibited; only occasionally was it suppressed. The duration of recovery in arterial pressure from these longer periods of obstruction is, however, definitely more prolonged. The phenomena are illustrated in fig. 7; in which the results of two of the experiments on dog 183 are recorded, results which have already been briefly reported elsewhere [Verney and Vogt, 1938 b; Verney, 1939]. In the one—continuous lines in the figure— the resting animal's pressure (open circles) was 120 mm. Hg. Fifteen minutes after zero time, 300 c.c. of water were given by stomach tube.

resting pressure was normal; and in animal 181, in which the resting pressure was raised, the latter effect disappeared, but the former persisted up to the time when the animal was killed. It seems to us that further insight into the nature of these apparently anomalous responses can best be obtained through the method of the renal artery loop,· recently described by Lockett, O'Connor, and Verney [1942]. We are here content to record the phenomena, leaving their proper interpretation to the results of future work.

When, however, the effects of partial but prolonged obstruction of the renal artery are investigated, they are seen to be much more uniform and intelligible than those just described. To these we now propose to turn.

The Effects of Prolonged but Partial Obstruction of the Renal Artery.

The observations were made on six dogs, Nos. 208, 206, 183, 238, 257, and 258, the effects of short occlusions of the renal artery in Nos. 183 and 208 having already been described.

In fig. 6 are given the results of two experiments on dog 208, those on the left having been obtained five days, those on the right twelve days after those recorded at J and K in fig. 4. At O (fig. 6) the renal artery was occluded for 120 sec.: during the next two minutes the urine flow was zero, and it then rapidly rose towards its value before the occlusion. At C the renal artery was partially obstructed: the urine flow was strongly inhibited, and remained so until the arterial obstruction was released at R; it then mounted rapidly, the increase in rate being observed as early as 30 sec. after the release. A rise in arterial pressure was detected 11 minutes after the renal artery was obstructed, and a plateau was reached about 15 minutes later. The pressure persisted at this raised level for 5 minutes after the release of the obstruction, but 10 minutes later it had definitely fallen, and 15 minutes after this it had attained its normal value. Two days later, the kidney was freed from its bed by dividing between ligatures all its attachments other than the renal artery, renal vein, and ureter; and it was then restored to its original position by tying together each pair of ligatures between which the perirenal tissues had been severed. This ensured the division of all ancillary vessels and of any renal sympathetic nerves which were not divided when the unit was implanted, or had since regenerated. Five days after this operation, an experiment similar to that illustrated in the left part of fig. 6 was performed, and closely comparable effects were observed: they are shown in the right part of the figure. The results of all experiments comprising partial obstruction of the renal artery in this animal (208) after the prolonged effects of short occlusion had disappeared, are collected in Table I. The figures show that partial obstruction of the renal artery for periods of about 40 minutes is accompanied by an increase in arterial pressure,

Animal / Type	Date							Time	Remarks
206 Type I.	21.11.38	70	30	165	150	,,,	,,,	7 hr.	mained so during the ... as the animal was returned to its was not followed subsequently, as the animal was returned to its kennel and the B.P. determined at intervals of about 40 min.
183 Type I.	18.5.38	50	30	128	124	..	152		Urine flow suppressed during period of compression, and began to recover 6 min. after the release, the centrifuged urine showing r.b.c. and a few hyaline casts.
	20.5.38	40	40	420	124	18	155	7 to 14 hr.	Urine flow strongly inhibited, but not suppressed.
	21.5.38	40	30	170	120	12	160	9 hr.	Urine flow strongly inhibited, but not suppressed. This experiment is illustrated in fig. 7.
	24.5.38	30	30	165	120	18	135	..	Urine flow inhibited, but not suppressed. Immediately after the release, a little larger volume of fluid was injected, with the results shown in the next row.
	24.5.38	40	25	120	134	(20*)	150	4 hr.	Just before the injection, the urine flow was 2 c.c./15 min. For the 10 min. following the injection, the average rate was 1·2 c.c./15 min.: the observations on urine flow were then discontinued.
									* The time at which a rise in B.P. occurred was difficult to determine, as the animal was rather restless for 10 min. after the mine, as the animal was rather restless for 10 min. after the injection. This experiment is illustrated in fig. 7.
	25.5.38	30	? 20	206	120	9	150	7 hr.	Urine flow suppressed for at least 40 min. after injection; the observations on urine flow were then discontinued. 2½ hr. later, 40 c.c. urine were obtained from the bladder by catheter, the urine containing albumen; and during the next hour 37 c.c. urine were collected, the urine containing albumen and a few casts and, in the early specimens after release, fat.
			Averages	203	124	14	150	7–8 hr.	
238 Type II.	27.7.39	90	40	45	155	6	180	20 min.	Continuous record of B.P. under local anesthesia from branch of femoral artery. Urine flow not observed. Both kidneys present.
	29.7.39	100	60	30¼	138	6	174	25 min.	Ditto. This experiment is illustrated in fig. 8.
			Averages	38	146	6	177	23 min.	
257 Type III.	16.1.40	..	..	23	110	6	136	..	Ditto. Recovery of B.P. not recorded, as animal became restless 7 min. after the release of the compression. The B.P. was normal when taken 2½ hr. later.
258 Type III.	2.2.40	..	..	35	110	..	..	..	Ditto. No change in arterial pressure.
	8.2.40	..	..	29	116	..	..	..	Ditto. No certain change in arterial pressure.
	12.2.40	..	..	24	110	..	..	..	Ditto. No certain change in arterial pressure.

Number of dog and type of unit implanted. For operative histories see pp. 30–40.	Date of experiment.	Vol. liquid injected into and recovered from compression unit: c.mm.		Period of compression: min.	Resting B.P. mm. Hg.	Time after compression at which a rise in pressure was first detected: min.	Max. B.P. during period of compression: mm. Hg.	Period of recovery of B.P. after release of compression.	Remarks.
		Injected.	Recovered.						
208 Type I.	6.3.39	70	60	(30)	(115)	..	..	..	This compression produced no inhibition in urine flow and no rise in B.P. After the release, a little larger volume was injected, with the results shown in the next row.
	6.3.39	80	70	34	115	9	153	40 min.	Urine flow suppressed, except for an occasional drop, during period of compression. It began to recover 3 min. after the release and rapidly increased, the first few samples containing fat and albumen.
	8.3.39	80	?	(< 62)	118	8	142	60 min.	Urine flow inhibited for 10 min, after which it was suppressed for 30 min. except for an occasional drop. It then rapidly increased, the first few samples containing fat. The unit had evidently leaked a little from its external end before the intentional release of the pressure.
	10.3.39	85	65	48	122	12	155	105 min.	Urine flow strongly inhibited during period of compression. Rapid increase beginning within 2 min. of release, the first few samples containing fat and a little albumen.
	13.3.39	75	50	54	112	11	143	30 min.	Urine flow strongly inhibited or suppressed during period of compression. Rapid increase beginning 30 sec. after release, the first few samples containing fat and a little albumen. This experiment is illustrated in fig. 6 (left).
	20.3.39	90	65	47	110	8	142	40 min.	Urine flow suppressed or strongly inhibited during period of compression. It began to recover 5 min. after the release, the first few samples containing r.b.c. and fat; albumen had not completely disappeared 40 min. after the release. This experiment is illustrated in fig. 6 (right).
	22.3.39	80	60	(31)	(114)	..	..	..	The compression produced only slight inhibition in urine flow, and no rise in B.P. After the release, a little larger volume was injected, with the results shown in the next row.
	22.3.39	85	65	25	114	10	140	40 min.	Urine flow strongly inhibited but not suppressed. Its rapid recovery began 4 min. after the release, the first few samples containing a little fat: albumen had disappeared 34 min. after the release.

longer period, or the rate of its inactivation or excretion is less than after ischæmia of shorter duration. These factors may share responsibility for the phenomenon, and the definition of their respective rôles must await chemical identification of the pressor agent, and methods for its estimation in the blood and urine.

The damage to the kidney by these degrees and durations of partial obstruction of the renal artery is not severe, since albumen has been detected in the urine for periods of not more than about 30 minutes after release of the obstruction. Blood has been observed only rarely in the centrifuged deposit; but fat, described elsewhere as appearing after short occlusions of the renal artery [Verney and Vogt, 1938 a], has been regularly seen as a cloud in the urine secreted just after release of the obstruction. No rise in pressure has been observed unless the urine flow has been at least strongly inhibited by the arterial obstruction.

It seemed to us that an indication of the part played by normal renal tissue in modifying the blood-pressure responses to temporary renal ischæmia might be given by determining the results of ischæmia of the one kidney when the other was *in situ*. In three animals, then (238, 257, and 258), the effects of partial obstruction of one renal artery were observed when the contra-lateral kidney was present. The splanchnic nerves on each side were divided, and the kidney to whose artery the compression unit was being applied was completely denervated. During the observations, the blood pressure was recorded by means of a cannula introduced, under local anæsthesia and with aseptic precautions, into a branch of the femoral artery. The results of these experiments are given in Table I, and illustrated by the tracing in fig. 8. As may be seen, the arterial pressure, which is initially steady at about 138 mm. Hg, is beginning to rise 6 minutes (b, fig. 8) after the commencement (first arrow) of the period of obstruction of the renal artery, and has reached a value of 155 mm. after 18, and 174 mm. Hg after 30 minutes of renal ischæmia. Ten minutes after release of the compression (second arrow), the arterial pressure is 162 mm. Hg, and it reaches its original level within the next 15 minutes. The latency of the rise in pressure recorded in two of these animals (238 and 257) is a little less than that observed by the carotid loop method in dogs 208, 206, and 183, and the degree of ischæmia was probably greater; but although the periods of ischæmia and the increases in blood pressure are similar to those in dog 208, the periods of recovery in the two experiments on dog 238 are somewhat shorter, a result which suggests that the normal kidney is excreting, inactivating, or depressing the formation of the pressor agent. Indeed, in the third animal (258) no change in pressure was observed from a degree of ischæmia which, in the absence of the contralateral kidney, would probably have resulted in hypertension. The figures are too few to warrant conclusive statement, and it seems to us that this can best be reached through the method of the renal

At C_1 40 c.mm. fluid were injected into the compression unit; 12 minutes later the pressure was rising, and it reached a value of 160 mm. 70 minutes after the compression of the renal artery was begun. The urine flow (black circles) was strongly inhibited, but was not suppressed. At R_1 the renal artery was decompressed, and the arterial pressure gradually fell to reach its normal value about 9 hours later. In the other experiment—broken lines in the figure—250 c.c. of water were given by stomach tube 195 minutes and 300 c.c. 50 minutes before zero
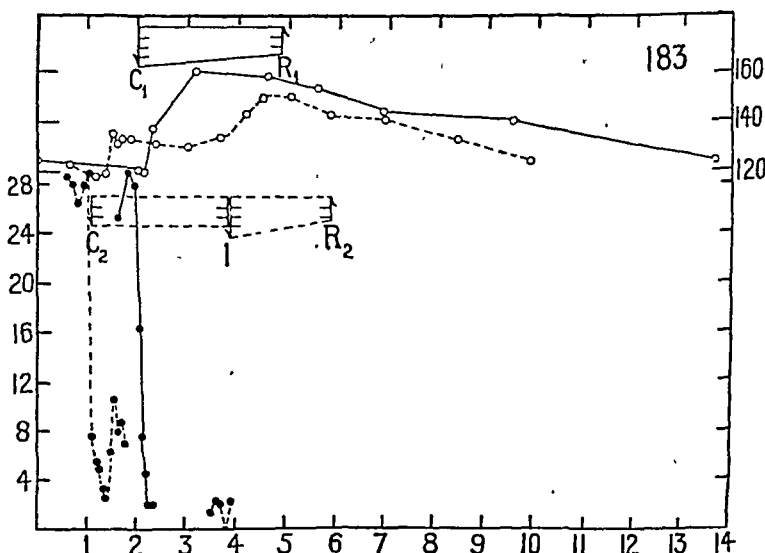
FIG. 7.—The effects of partial obstruction of the right renal artery in dog 183 on 21.5.38 (continuous lines) and on 24.5.38 (broken lines). Ordinates: left, urine flow in c.c./15 min.; right, blood pressure in mm. Hg. Abscissæ: time in hours. For further description, see text.

time. The resting animal's pressure (open circles) was again 120 mm. Hg. At C_2 30 c.mm. of fluid were injected into the compression unit; 18 minutes later the pressure was rising, and it reached a value of 135 mm. 30 minutes after the compression of the renal artery was begun. The urine flow (black circles) was considerably reduced. At I the renal artery was decompressed, and immediately afterwards 40 c.mm. of fluid were injected into the compression unit. A further rise in arterial pressure followed this manœuvre; and after the renal artery was finally decompressed (R_2), 4 hours elapsed before the arterial pressure had fallen to its normal value. The much longer periods of recovery in these animals, compared with those observed in dog 208, are apparently due to the longer periods of ischæmia to which the kidneys have been subjected. We infer, therefore, that under such circumstances either the production of the pressor agent by the kidney is continued for a

rises in blood pressure. We hoped to obtain this information by temporarily obstructing the blood supply to kidneys which had been transplanted to the neck, the pressure in the renal artery thereby becoming susceptible to direct measurement. Braun-Menendez and Fasciolo [1940] have recently reported that obstruction of the arterial supply to a kidney grafted to the neck of a chloralosed and nephrectomised dog produces, in 2 to 7 minutes, an increase of 8 to 30 mm. Hg in the arterial pressure, an increase which subsides when the obstruction is released.

In our experiments the kidneys were taken either from chloralosed or from decerebrated donors and grafted to the necks of animals which had been decerebrated under ether anæsthesia, the period of anæsthesia having been about 20 minutes, and at least 2 hours having elapsed between the decerebration and the transplantation of the kidneys. The neck of the recipient was prepared by dissecting one carotid and one jugular vein, or both carotids and jugulars, and fitting the vessels with Payr's cannulæ. The kidneys were obtained from the donors in one of two ways. In the one, they were freed from all connexion with the body other than the renal artery and vein and the ureter, rapidly excised, and the vessels were immediately anastomosed with the neck vessels of the recipient. In the other, the animal was eviscerated, and the aorta and inferior vena cava were mobilised by tying and dividing all their branches and tributaries between the suprarenals above and the pelvis below, other than one or both renal arteries and veins. The posterior ends of the aorta and cava were then anastomosed with the carotid artery and jugular vein of the recipient, and the transplantation was completed by dividing between ligatures both the aorta and the cava anterior to the renal vessels. This procedure has been described by Govaerts [1929], and while it involves more handling of the kidney than does the former method, interruption of the renal blood supply is avoided. In some experiments a kidney was transplanted to the neck of the same dog by the former of the two methods. The renal blood flow was reduced by compressing the anastomosed carotid by means of a mechanical device. The renal arterial pressure was measured by a membrane manometer connected with the anterior end of the excised aortic segment, and the general arterial pressure by a mercury manometer connected with the femoral artery.

Six such experiments were made with the following results. In four nephrectomised recipients, severe obstruction of the blood supply to the transplanted kidney produced small rises in arterial pressure, viz. between 7 and 18 mm. Hg. In one of these animals the anastomosed carotid was compressed at different times after the nephrectomy: when this was done a few minutes after the removal of the kidneys, no rise in pressure occurred, but rises were observed when it was repeated 2 and again 4 hours later. The smallest effect was obtained by obstructing

artery loop [Lockett, O'Connor, and Verney, 1942], in that a greater and more enduring control over the degree of arterial obstruction is
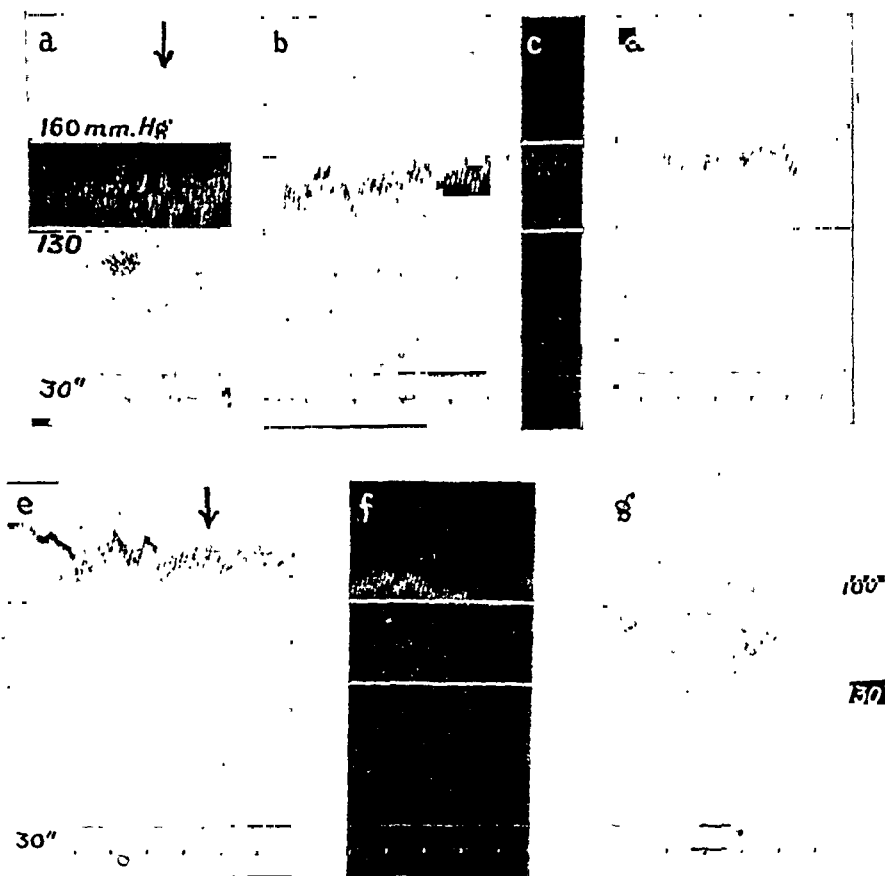
FIG. 8.—Femoral arterial pressure in dog 238 on 29.7.39. At the arrow in *a* the right renal artery was compressed, and the compression was released at the arrow in *c*. Between *a* and *b*, *b* and *c*, *c* and *d*, intervals of 4 minutes; between *d* and *e*, *e* and *f*, intervals of 8 minutes; between *f* and *g*, an interval of 12 minutes. Time signal: 30 sec. The dog was asleep during the first 56 minutes of the blood pressure record, and apparently quite unaware of the compression and decompression of the renal artery: it awoke during the tracing *g*. For further description, see text.

offered by this than by the methods through which the results here described have been obtained.

Experiments on the Production of Hypertension by obstructing the Renal Arterial Supply in non-surviving Animals.

The experiments so far described give no information on the degree of reduction in renal arterial pressure needed to elicit rapidly occurring

responses obtained in the living animal. A further resemblance lay in the absence of response when the recipient had not been deprived of its own kidneys: in the survival experiments the effects on the blood pressure of compression of a renal artery in the presence of the contralateral kidney were, as already reported, probably smaller than in its absence.

The foregoing observations, then, have shown that local reduction in the pressure of blood supplied to a kidney transplanted from a chloralosed or decerebrated donor to the neck of a decerebrated and nephrectomised recipient, produces a rise in the general arterial pressure as rapidly as does compression of the renal artery by means of a unit implanted in the living dog. The degree of rise in pressure is, however, much less; and it is unknown whether this is due to the abnormal condition of the kidney, of the recipient, or of both. The smallness of the responses renders the method unsuitable for the provision of accurate information on the conditions of their production: their occurrence with transplanted kidneys, however, removes doubt as to the humoral nature of the phenomenon, and suggests, therefore, that the cause of the rapid blood-pressure response to compression of the renal artery by means of an implanted unit is the same as that of the persistent hypertension from permanent obstruction of the renal artery by operative procedures, a phenomenon which has been shown to be of humoral origin.

We mentioned above that in a single experiment in which the arterial pressure was raised by obstructing the blood supply to a transplanted kidney, the pressure returned to its original level within 12 minutes of the removal of the ischæmic kidney. Since the animal had no other kidney, the pressor agent was evidently being inactivated in the body. Similarly, extirpation of a chronically ischæmic kidney is followed by a gradual fall in blood pressure to the normal value, whether or no the animal is in possession of its other kidney [Rodbard and Katz, 1939]. We, too, have observed such a fall to result from functional exclusion of the sole remaining kidney in an animal hypertensive from renal ischæmia. In this animal [No. 171, see Verney and Vogt, 1938 b, pp. 269–270], the posterior primary branch of the left renal artery was tied at a first operation, but the ancillary supply and drainage were left intact. Thirty-five days later the ancillary vessels and further branches of the left renal artery were tied, and a month later the right kidney was removed. The resultant hypertension gradually decreased, doubtless owing to the redevelopment of an ancillary blood supply, and at the end of three months the arterial pressure was 136 mm. Hg. A further operation was then performed, consisting in the implantation of a fine Bowden tube [Verney, 1930] protected externally by a thin rubber sheath, and containing a central thread the end of which was looped around the renal artery; the whole ancillary supply and drainage was tied. As a result of this operation the arterial pressure, measured

one renal artery after transplanting a pair of kidneys to the neck; and in three control experiments in which the animals were in possession of their own kidneys, or at least one of them, no change in arterial pressure followed obstruction of the arterial supply to the transplanted kidney.

The time-course of the effects produced by ischæmia of a transplanted kidney is illustrated by the data in Table II. These were obtained from a dog decerebrated 6 hours and nephrectomised unilaterally 2 hours before the observations were begun; the dog's second kidney was transplanted to the neck 100 minutes before the first

TABLE II.

	Time.	B.P. mm. Hg.
Obstruction of blood supply to kidney: urine flow suppressed	6.06 p.m.	138
Rise in B.P. becoming significant . .	6.12 ,,	144
Maximum B.P. reached	6.27 ,,	156
Obstruction released	6.41 ,,	156
Return of urine flow	6.48 ,,	148
Original level of B.P. reached . .	7.01 ,,	138

arterial compression. In this experiment the pressure was rising significantly within 6 minutes, the maximum was attained in 21 minutes, and approximately the same period elapsed between the release of compression and the return of blood pressure to its original level. The urine flow began to recover 7 minutes after the release, and at this time a significant fall in arterial pressure had already appeared.[1] Similar results were obtained when, 13 minutes later, this experiment was repeated. Instead, however, of releasing the arterial obstruction when the plateau of raised pressure was reached, we removed the ischæmic kidney: the blood pressure fell even more rapidly than in the previous experiment, and had reached its original level within 12 minutes.

In order to produce rises in blood pressure in these non-survival experiments, the obstruction of the renal artery needed to be so severe as to suppress the urine flow: suppression or strong inhibition of urine flow also occurred, as reported earlier in this paper, in experiments on the surviving animal. Indeed, the pressure in the renal artery was reduced to values between one-half and one-third of the original arterial pressure. When the compression was released, the urine flow recovered within a few minutes, and fat was observed in the urine as in the survival experiments. There was no difficulty in reproducing these effects several times, and their time-courses were similar to those of the

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[1] The results given in fig. 6 (right) of a survival experiment on dog 208 show time-courses of blood pressure and urine flow in close accord with these.

after an incision had been made along each, the veins were anastomosed by two continuous sutures of very fine silk sterilised in vaseline. On release of the clamps, any small ooze of blood from the site of the sutures was arrested by fine ligature, and the thread which had been previously looped around the anterior end of the abdominal cava was tied. The renal venous blood was now flowing entirely into the portal vein. In some animals transient œdema of the hind limbs resulted from this operation, and the rate of healing of the abdominal wounds was retarded.

After a period which varied between six and nineteen weeks, one or both renal arteries were obstructed by the method described elsewhere [Verney and Vogt, 1938 *b*]; at the same time any renal venous collaterals which had developed since the last operation were carefully sought and tied.
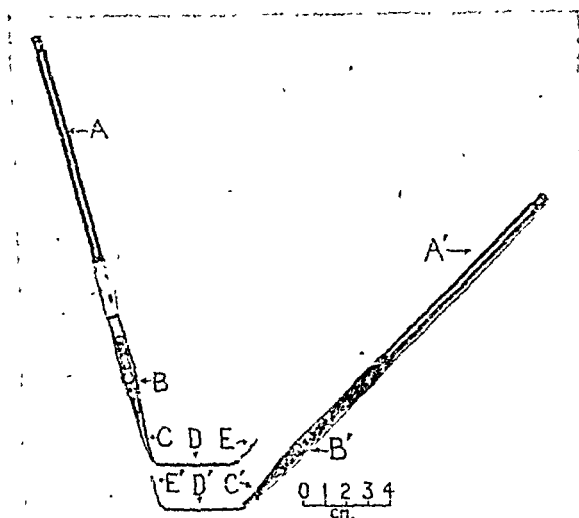
FIG. 9.—Miniature "gastro-enterostomy" clamps for securing and approximating the folds of the portal and inferior caval veins during the formation of the venous anastomosis. A: handle, B: fixing slide, C: eyelet, D: blades, E: points of caval clamp. A', B', C', D,' and E': corresponding parts of portal clamp.

Three dogs survived these stages, and when at the conclusion of the observations the animals were killed, post-mortem examination revealed a widely patent anastomosis as the sole pathway for the renal venous blood. Fig. 10 illustrates the course of the arterial pressure in one of these animals before and after the production of renal ischæmia. During the preliminary period the systolic blood pressure was about 120 mm. Hg. At A the right renal artery was slightly obstructed by means of a silver clip of 7 mm. length and 2·5 mm. internal diameter, the kidney was lifted from its bed, and all its ancillary vessels were divided between ligatures. The operation was followed by a rise in arterial pressure of 30 mm. Hg, a rise which decreased to 20 mm. during the next twenty-eight days. A similar operation was then performed on the left kidney (B, fig. 10); the obstruction, however,

by the method of the denervated carotid loop, rose to 190 mm. Hg.
Three days later, the blood pressure being still 190 mm., the thread in
the Bowden tube was pulled taut, thus occluding the renal artery.
This manœuvre was followed by a fall in arterial pressure, and the pre-
operative level of 136 mm. Hg was reached in 3 hours 42 minutes and
was maintained for the ensuing 16 hours. The hypertensive agent,
then, is inactivated somewhere in the blood or tissues, and it seemed to
us of interest to inquire whether the liver played a predominant rôle
in this inactivation.

Experiments on the Effects of Renal Ischæmia in Animals in which the Renal Venous Blood is conducted directly into the Portal Vein.

We thought that if the liver played such a rôle, the conduction of
the renal venous blood directly into the portal vein might produce
conditions in which subsequent renal ischæmia was not followed by
hypertension. The plan of the experiment, therefore, was, first, to
establish a collateral venous return from the tissues posterior to the
kidneys by occluding the vena cava posterior to the renal veins; second,
to anastomose the portal vein with the renal segment of the vena cava,
and to tie the cava anterior to the anastomosis as well as all ancillary
renal venous channels; and third, to produce ischæmia of the kidneys
by the application of silver clips [Verney and Vogt, 1938 b] to the renal
arteries, and at the same time to search for and tie any newly developed
venules carrying blood from the kidneys to the systemic circulation.

Details of the procedures are as follows. A carotid loop with denervated
sinus was made in each bitch at a preliminary operation. Since occlusion
of the inferior vena cava at a single operation had proved fatal in a certain
number of animals, the lumen was diminished at a first operation by Theobald's
[1931] method. Under the vessel was passed a short length of fine rubber
tubing with a central thread, the tubing being of such length that when the
thread was tied the lumen of the cava was reduced to a diameter of 3 or 4 mm.
The cava was thus obstructed about midway between the renal and the iliac
veins. Œdema of the hind limbs often ensued, but, as the result of the growth
of venous collaterals, it had always subsided when, at a second operation
two or three weeks later, the cava was occluded at the same level.

After the animal's complete recovery, the anastomosis operation was
undertaken. The cava was tied and divided just posterior to the renal veins,
all tributaries other than these, up to the level of the diaphragm, were cut
between ligatures, and a loose thread was passed around the vessel well anterior
to the renal veins. Several centimetres of the portal vein were then freed
from connective tissue, and two clamps [1] (fig. 9) were placed the one on a fold
of the vena cava, the other on a fold of the portal vein. The clamps were
brought together, and held in apposition by inserting the points E, E' through
the eyelets C', C respectively. The length of the folds was about 3 cm., and

cardiac, and gastro-intestinal hæmorrhages were among the post-mortem findings.

The effects of renal ischæmia in these animals are, then, so far as we have been able to determine, identical both in kind and in degree with those obtained in normal dogs from similar degrees of obstruction of the renal arterial supply. Were the liver to play an elective rôle in the inactivation of either the pressor agent or, if we assume these substances not to be identical, the agent responsible for the syndrome of convulsive "uræmia," we might expect their direct conduction to the liver to be associated with their greater inactivation, and the systemic effects of these substances to have been correspondingly smaller. This expectation has not been fulfilled. Child and Glen [1938] made a reverse Eck's fistula in two dogs previously rendered hypertensive, and observed no lasting effect of this procedure on the dogs' raised pressure; furthermore, constriction of the renal arteries in two other of their dogs, in which the venous anastomosis had been made at a preliminary operation, was followed by rises in blood pressure similar to those observed in normal controls. Their evidence, however, is not entirely convincing, since no attempt was made to prevent the flow of renal venous blood through alternative channels, nor at the time of publication of their results had post-mortem examinations, which alone could show to what extent such ancillary routes were present, been made. Similarly, Levy and Blalock [1938] made an end to end anastomosis between the left renal vein and the splenic vein, "thus forcing a large part of the blood from the kidney to return to the heart by way of the liver," and several days later they removed the right kidney. They report that after the recovery of four animals from this operation, partial occlusion of the left renal artery by means of the clamp described by Goldblatt, Lynch, Hanzal, and Summerville [1934] was followed by a rise in arterial pressure as measured by femoral artery puncture: no post-mortem findings are described.

DISCUSSION.

There can be little doubt that the rise in arterial pressure, which we have shown to occur when the renal artery is partially and temporarily obstructed in the living dog, has the same immediate cause as the persisting hypertension which Goldblatt and his colleagues [1934] first observed to result from renal ischæmia. Both are humorally determined, and the technique of the compression unit has allowed the anæsthesia complication of surgical procedures to be eliminated, and the time-course of the rise in pressure to be followed. The fact that the pressure may be rising significantly some 6 minutes after the induction of renal ischæmia, and that the response is reversible, show that the pressor agent is one which is readily at call, and whose production

was more severe (diam. of clip, 2·25 mm.) than that previously induced
on the right side.   Renal ancillary vessels on both sides were carefully
sought and tied, this being necessary on the right side in spite of the
division of the ancillary vessels at the operation four weeks earlier.
The ensuing rise in arterial pressure was rapid and large, and 48 hours
after the operation the syndrome of convulsive "uræmia" [Goldblatt,
1938; Child, 1938; Verney and Vogt, 1938 b] began to appear.   On the
development of convulsions, vomiting and diarrhœa, the animal—it
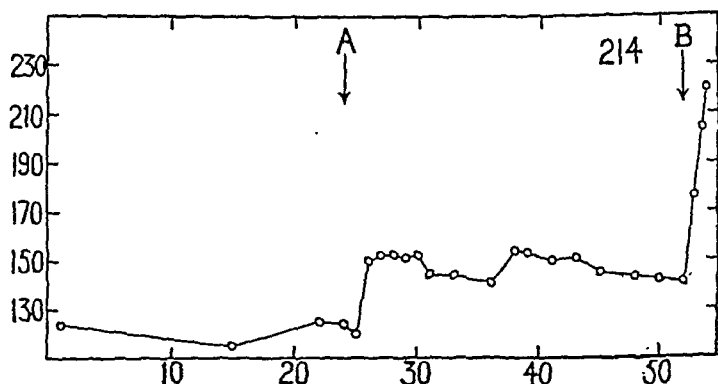
Fig. 10.—The effects of renal ischæmia on the systolic pressure of dog 214.
20.1.39: denervation of carotid sinus and formation of carotid loop.   16.2.39:
inferior vena cava obstructed.   27.2.39: inferior vena cava occluded.   17.3.39:
anastomosis between the renal segment of cava and the portal vein; at its
completion there was no congestion of the gut or of the kidneys.   3.4.39: date
of first blood-pressure record in the figure.   A: right renal artery obstructed.
B: left renal artery obstructed.   Ordinates: blood pressure in mm. Hg.
Abscissæ: time in days.   For further description, see text.

was not anuric—was killed with chloroform; and post-mortem examina-
tion disclosed hæmorrhages in the cardiac muscle, severe hæmorrhagic
lesions in the mucosa of the duodenum and upper jejunum, and milder
lesions in that of the large intestine.[1]

The results of similar procedures with the other two animals
corroborate those just described.   One reacted to mild obstruction of
its right renal artery by a rise in blood pressure of 20 mm. Hg.   The
other was subjected to simultaneous obstruction of both renal arteries.
On the fourth post-operative day the pressure, which in this animal was
measured under local anæsthesia by cannulation of branches of the
femoral artery, had risen from the pre-operative value of 96 mm. Hg
to 162 mm. Hg.   Convulsive "uræmia" developed, and on the fifth
post-operative day the dog was killed with chloroform.   Retinal,

_____
[1] It may be of interest to note that cardiac hæmorrhages, hyaline changes in
the arterioles and necrotic areas in the heart muscle, lesions which are seldom missing
in the dog when renal arterial obstruction is so severe as to be fatal, have recently
been described by Gouley [1940] as frequent accompaniments of uræmia in the
hypertensive man.

TABLE III.

Date and time.		Systolic B.P. mm. Hg.	Remarks.
13.11.39	10.15	133	
	10.30	136	
	10.45	..	Right renal artery occluded.
	11.13	136	
	11.35	145	
	12.00	148	
	12.25	152	
	12.45	151	
	14.20	154	Vomited.
	15.35	166	Vomited.
	18.23	196	
	21.10	206 to 212	
	22.50	200 to 210	
14.11.39	..	175 to 200	
15.11.39	18.00	190 to 230	Much vomiting during the day.
16.11.39	8.45	..	Clonic convulsions.
	9.40	140	
	10.35	144	
	15.30	..	Died.

was done, two showed no resultant increase in urine flow, and the remainder increases which, in 1 to 3 hours, reached values between 67 and 530 per cent. of the rates before the contralateral artery was occluded. The times at which an increase in urine flow was first observed varied between 15 and 80 minutes after occlusion of the artery. From examination of these responses, the conclusion was drawn at that time that their type was "similar to that observed when the kidney is subjected to a large increase in the pressure of its arterial blood supply." No measurements of the arterial pressure were, however, made; but it seems reasonable now to relate these delayed increases in urine flow with the delayed increase in arterial pressure which, as has been shown, is consequent upon occlusion of the main arterial supply to a sole remaining kidney.

Let us now consider whether the experiments reported in this paper shed any light on the fate of the pressor agent. We have seen that, in an animal hypertensive from renal ischæmia, the functional elimination of all renal tissue is followed by a gradual fall in arterial pressure (No. 171, see p. 56), the fall being presumably due to the destruction of the pressor agent at its site of action in the arterioles. The time-course of the fall in pressure in this animal is given in fig. 11, and to this have been attached the blood-pressure recovery curves of those animals in which hypertension was induced by temporary obstruction or occlusion of the renal artery. It is evident that in the majority of instances the rate of fall in blood pressure is greater than that exhibited

is initiated and suppressed by contemporary impairment and recovery of the renal circulation.

While the essential stimulus to the production of the pressor agent is clearly some change in the blood pressure–blood flow relations within the kidney, the consequent rise in arterial pressure is attributively conditioned by the intensity of the kidney's excretory load. The influence of dietary overload with meat, urea, or sodium chloride in augmenting the effects of renal ischæmia has already been demonstrated [Verney and Vogt, 1938 *b*]; and the fact reported above, that, while in a decerebrated and nephrectomised dog obstruction of the arterial supply to a transplanted kidney elicited no rise in arterial pressure when this obstruction was produced a few minutes after nephrectomy, it did so 2 and 4 hours later, is consistent with the response of the living animal to dietary overload on a background of renal ischæmia.

Whether the essential factor of arterial obstruction, and the ancillary factor of excretory load summate to produce within the kidney the immediate stimulus, *e.g.* oxygen want, to the formation of the pressor agent, or whether the latter factor operates exclusively on the peripheral vasculature, is as yet undetermined; though the former view would seem to contain the more likely explanation. The time-course of the rise in arterial pressure following obstruction of the renal artery would, however, be expected to be conditioned by the rate at which the pressor agent reaches the general circulation, and this in turn to be maximally determined by an optimal relation between intensity of local production and rapidity of peripheral distribution. Extreme degrees of renal ischæmia are, indeed, associated with long delay in the pressor response. This is illustrated by the following protocol.

Dog 220. 9.5.39: denervated carotid loop made. 14.–17.10.39: systolic pressure varied between 125 and 133 mm. Hg. 18.10.39: implantation of Bowden cable [Verney, 1930] dorsal to right renal artery, the thread being loosely looped around the artery near its origin, and there being no interference with the ancillary blood supply to the kidney. The B.P. rose a little and reached 150 mm. Hg four days after the implantation, remained at that figure for five days, and then gradually fell to reach 125 mm. on 2.11.39. 7.11.39: left kidney excised by lumbar route. 8.–12.11.39: systolic pressure varied between 125 and 140 mm. Hg. The following observations were then made (Table III).

In this experiment the blood supply to the kidney was suddenly reduced to that reaching it through the ancillary arteries, and some 50 minutes elapsed before the arterial pressure began to rise. In this connexion it is of interest to recall some earlier experiments [Verney, 1930; see fig. 3 in that paper] in which an increase in the flow of urine from one kidney was seen to follow occlusion of the main arterial supply to the other. The artery was occluded by the Bowden cable technique at times varying in the different animals between two and five days after implantation of the cable; and of the eight animals in which this

the recovery curve of animal 171 is lowered throughout by 15 mm. so as to bring its final plateau to a more normal resting value. It should be emphasised, however, that the deductions are highly speculative, in that they are derived from the synthesis of the courses of blood pressure in several animals: their correction or modification must await the isolation of the pressor agent and the elaboration of methods for its estimation in physiological fluids.

Although it is premature to offer explanation of the prolonged anuria and hypertension seen in three of the dogs submitted to short occlusion of the renal artery, an observation by Schroeder and Steele [1940] may be of interest in connexion therewith. They found that in the dog anæsthetised with pentobarbital sodium, the renal vessels became supersensitive to adrenaline when the renal artery was severely constricted, the vessels then responding to normally subeffective amounts by long lasting constriction. A supersensitivity of this nature may be operative in the prolonged responses which we have seen to follow short occlusion of the renal artery in some of our animals, though the conditions under which such occlusion would lead to the release, at the endings of renal adrenergic nerves, of effective amounts of adrenaline still remain obscure.

SUMMARY.

1. Methods for producing temporary obstruction, complete or partial, of the renal artery in the dog are described.

2. Occlusion of the artery to a sole remaining kidney for periods ranging between 2 sec. and 600 sec. produces, subject to the reservation in paragraph 3 below, contemporary suppression of urine flow without accompanying or subsequent change in arterial pressure as measured by the method of the carotid loop with denervated sinus.

3. In some animals long lasting suppression of urine, and hypertension, have been observed to follow short periods (from 5 to 120 sec.) of arterial occlusion (figs. 4 and 5). These responses resemble those accompanying prolonged but partial obstruction of the renal artery (see paragraph 4 below). The conditions of their occurrence are not known.

4. When the artery to a sole remaining kidney is partially obstructed, the arterial pressure is rising some 8 to 18 minutes later towards a plateau from which it slowly falls when the obstruction is released. The results suggest that the period of recovery in blood pressure is a function of the period of arterial obstruction (Table I). They occur whether or no the splanchnic and the renal nerves have been divided. No rise in pressure has been observed unless the urine flow, during water-diuresis, has been at least strongly inhibited by the arterial obstruction.

5. When the renal artery on one side is partially obstructed, the contralateral kidney being present, either no rise in arterial pressure

by the anephric animal, a result which suggests that the kidney, on release of its arterial obstruction, is engaged in excreting or otherwise disposing of the pressor agent which it was previously elaborating. In a few instances, however, the rate of fall in blood pressure is less than that exhibited by the anephric animal: in all these, the period $(2\frac{3}{4}$ to
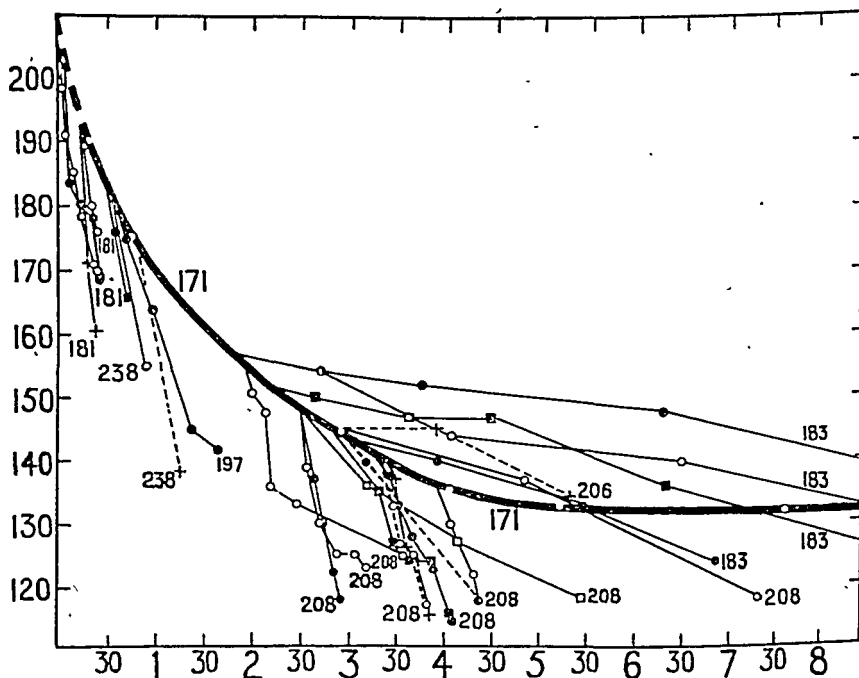
Fig. 11.—The heavy black curve represents the time-course of the fall in arterial pressure in dog 171 after functional exclusion of all renal tissue. To this have been attached the recovery curves of animals 181 (5), 238 (2), 197 (1), 208 (11), 206 (1), and 183 (4) after obstruction of the renal artery. In No. 197 (not previously mentioned in the text) the right renal artery was partially obstructed for 29 minutes by means of a compression unit, type I. The urine flow, which was suppressed by the obstruction, rapidly recovered 4 minutes after the release. The right kidney was denervated and the left removed when the unit was implanted. Ordinates: blood pressure in mm. Hg. Abscissæ: time in minutes and hours.

7 hours) of preceding compression of the renal artery, or the period (more than 3 hours) of suppression of the urine after short occlusion of the artery, was longer than in those exhibiting an increased rate of fall; a result which suggests that here the kidney is continuing the liberation of the pressor agent well into the period of recovery in blood pressure.[1] Such representation of results is not formally affected if

[1] In acute experiments on the anæsthetised dog, Friedman, Sugarman, and Selzer [1941] report that a long lasting reduction in renal blood flow, as measured by diodrast clearance, follows the release of compression of the abdominal aorta anterior to the renal arteries.

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ensues, or a rise from which recovery occurs apparently more quickly than in comparable experiments in which the contralateral kidney is absent.

6. The view that the increase in arterial pressure referred to in paragraph 4 above is of humoral origin, is confirmed by the occurrence of a similar phenomenon in preparations in which a kidney has been transplanted to the neck of a decerebrated and nephrectomised dog. Either release of the obstruction, or removal of the kidney, is followed by a gradual return of the arterial pressure to its original value. In order to produce increases in general arterial pressure in such preparations, the reduction in renal arterial pressure needs to be severe: the resultant increases in general arterial pressure are, notwithstanding, much smaller than in survival experiments.

7. The results suggest that the cause of the rapid blood-pressure response to compression of the renal artery by means of a previously implanted unit, is the same as that of the persistent hypertension which follows permanent obstruction of the renal artery by operative procedures.

8. Functional removal of a chronically ischæmic and sole remaining kidney was followed by a gradual fall in arterial pressure. The hypertension had disappeared within 4 hours, and thereafter the pressure remained constant for 16 hours.

9. Animals in which an anastomosis has so been made between the renal segment of the I.V.C. and the portal vein that all the renal venous blood is conducted directly to the liver, respond to the induction of renal ischæmia in ways similar to those in which normal animals respond. It is improbable, therefore, that the liver plays an elective rôle in the inactivation of either the pressor agent or, if we assume these substances not to be identical, the agent responsible for convulsive "uræmia."

10. Consideration of the results reported in this paper shows them to be compatible with the view that normal renal tissue both depresses the rate of formation of the pressor agent by ischæmic renal tissue, and accelerates, by a process of excretion or inactivation, the rate of its disappearance from the blood; further, that when the period of renal ischæmia is long (*e.g.* 3 hours) the production of the pressor agent continues for some hours after the mechanical release of the obstruction to the renal artery.

EXPERIMENTAL THERMAL BURNS, ESPECIALLY THE MODERATE TEMPERATURE BURN. By E. H. LEACH, R. A. PETERS, and R. J. ROSSITER. From the Departments of Biochemistry and Physiology, Oxford.

(A) INTRODUCTION AND BURNING IRON DEVICE, by R. A. PETERS.

(B) MACROSCOPIC AND MICROSCOPIC DAMAGE AT MODERATE TEMPERATURES, by E. H. LEACH (responsible for histological work), R. A. PETERS, and R. J. ROSSITER.

(A) INTRODUCTION AND BURNING IRON DEVICE.

Introduction.—Two schools of thought exist in regard to the so-called toxæmic effects in burns. The one is represented, among others, by Robertson and Boyd [1923] and by Wilson and colleagues [1937–38], who think that there is a toxæmic factor apart from infection. The other view was taken especially by Underhill and colleagues [1930] in their series of careful papers; they thought that the toxic effects could be explained by the blood concentration. Colour is undoubtedly given to this interpretation of the phenomena by the recent work of Florey and colleagues [Abraham, E. P., *et al.*, 1941] on shock; these authors have proved that the toxæmic effects hitherto thought to be due to tissue constituents from minces of liver introduced intraperitoneally are absent when bacteria have been excluded.

In planning the experiments here to be reported, we have been guided by the idea that any toxæmic effects which may be present, apart from those due to the blood concentration and to the growth of bacteria, must be derived from the area of partly damaged tissue rather than from that which has been completely coagulated.

Consider the following diagram of a burn. (Fig. 1.)

I is an area of fully heated tissue to which enough heat has been applied to fix the tissue proteins. II is an area of varying depth to which a heat gradient of varying temperature has been applied, extending from that of the fully burnt area to that of the body temperature, $37°$ C. So far, much thought has been concentrated upon I; but the most likely site for the origin of dangerous toxins is II; here it may be expected that, owing to the more moderate temperature reached, sufficient injury to the tissues will have been done to damage cell

of the apparatus, which is circular and made of brass, the surface for application being tinned to prevent contact of the copper. The sides are completely lagged with asbestos to prevent loss of heat and to limit the heat of application. The temperature of water running through can be adjusted simply; if this flows from an ordinary Fletcher gas heater, temperatures of 50°–55° C. can be maintained within 0·2° C.

FIG. 2.

It has proved possible to get standard moderate temperature burns with this apparatus, and so to study graded temperature effects properly. This apparatus is similar in principle to the small glass bulbs used by Hudack and McMaster [1932] for heating mouse ears, though it was designed independently some time before acquaintance with their paper. It differs in size and in the possession of the thin metal tinned surface, which is likely to give a better conduction of heat.

(B) MACROSCOPIC AND MICROSCOPIC DAMAGE AT MODERATE TEMPERATURES.

Historical.—As indicated in the introduction, we set out to find the relation between temperature, time of exposure, and skin damage, and have found that for external applications 50° C. is a critical temperature with the heating iron described. Subsequent search of the literature showed that very few attempts had been made to produce graded moderate temperature burns in animals; some of the most accurate, with which our observations on ordinary skin are in agreement, were made 70 years ago by Cohnheim [1873], who temporarily stopped the circulation in rabbits' ears with ligatures and then placed the ears in a water-bath at an observed temperature. He found that immersions of 30 minutes with the water at 45° C., and of 6 to 7 minutes with the water at 49° C. produced no effect. An immersion of 30 minutes at 49° C. produced transient redness and swelling, and at all temperatures greater than 50° C. severe damage was done. An immersion of 6 to 7 minutes at 52° C. gave a patchy partial necrosis, while the same exposure to 60° C. gave complete necrosis. Similar results are reported by McMaster and Hudack [1934] for mice ears;

permeability without altering the chemical nature of some proteins and enzymes. At least two things may then happen. Some cell constituents may diffuse out of the partly damaged cells. If these happen to be active enzymes or potentially dangerous substances, their presence in parts of the body to which they are foreign may cause toxic effects. Alternatively, destruction of the enzymes more sensitive to heat, leaving others intact and active, may upset the cell balance so as to cause the active production of toxins. In any case, the effects cannot be predicted without further knowledge.[1]

Exploring the view that toxæmic effects in burns may be specially related to the moderately heated area,[2] we should look for the "burn"
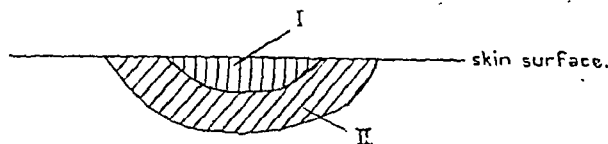
FIG. 1.

toxin (a) by burning animals with temperatures low enough to cause cell damage without destroying all the cell proteins; (b) by finding out which enzymes and tissue constituents stand heating to the appropriate temperatures; and (c) perhaps even by injecting into animals extracts made under similar conditions from skin and tissues.

An attempt to test these views experimentally revealed a singular lack of fundamental knowledge in two directions. There was really no answer to the question what exposure to heat and at what temperature will produce skin damage, and there was little systematic information upon the temperatures to which the enzymes in cells can be subjected without damage. Both these questions have to be answered in the rough before more detailed biochemical work can proceed. We deal with the first here; the apparatus described below has been designed to study the minimal temperatures causing tissue damage.

Simple Heating Iron for applying Temperatures up to 80° C. to the Skin (devised with the mechanical assistance of J. Cox).—The apparatus used is represented in the diagram (fig. 2). This shows a cross-section

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[1] It is relevant to this line of thought that some surgeons excise not only the actual burnt area, but a substantial margin of tissue which is believed to be damaged upon histological grounds. The whole issue seems fundamental to treatment. Supposing that bacterial invasion can be completely prevented, what will then happen to the area of partly damaged tissue? Can it be left for the leucocytes to remove, or will it contribute to an unpleasant slough, which should not be left enclosed under a film or other dressing? If there is a toxic agent soon liberated from a burnt area, we ought to direct further attention to its immobilisation in any first aid treatment; this should not prove an impossible biochemical task, if we have a better idea of its chemical nature.

[2] We have not seen this view expressed before, though doubtless it has been in the minds of some workers.

of the apparatus, which is circular and made of brass, the surface for application being tinned to prevent contact of the copper. The sides are completely lagged with asbestos to prevent loss of heat and to limit the heat of application. The temperature of water running through can be adjusted simply; if this flows from an ordinary Fletcher gas heater, temperatures of 50°–55° C. can be maintained within 0·2° C.
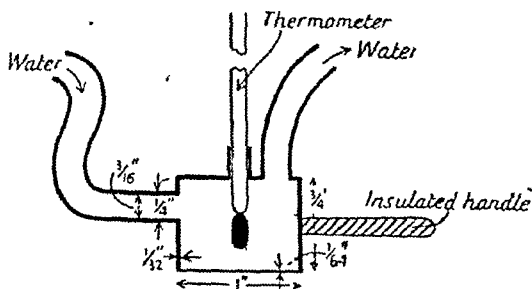

Fig. 2.

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the primary interest of these authors lay in the lymphatics. They found that 1 minute at 59° C. to 60° C. and 20 seconds at 67° C. gave necrosis, while 1 minute at 55° C. produced a moderate burn.

There is also some information available as to the temperature at which certain of the tissues are killed. For instance, Moore [1924] found that the temperature of the abdominal cavity of rats, guinea-pigs, and rabbits produced a degeneration of the germinal epithelium of the testis. Warming the testis to 47° C. for 5 minutes by the application of heat externally produced the same result. Hart [1922] and Stieve [1923] both found that hyperthermia rendered mice sterile. Pincus and Fisher [1931] reported that temperatures between 44° C. and 47° C. kill chick osteoblasts in culture, the exposure time necessary being dependent upon the temperature, and Kokatt [1930] found that temperatures from 42° C. to 50° C. inhibited mitoses in tissue cultures. Schultze [1865] found that whereas 45° C. to 46° C. produced no effect on leucocytes, 50° C. killed them. Red cells tolerated 52° C. Siebert [1928] reported that cartilage transplants did not grow if heated for longer than 30 minutes at 47° C. Much work has also been done on the effect of temperature on transplants of tumour cells. Typical of such reports are those of Loeb [1903], Lambert [1912], and Stevenson [1919]. Temperatures of 47° C. appear to kill all types of cells, and between 42° C. and 47° C. death of cells can be produced if the time of heating is long enough. The order of heat sensitivity seems to be sarcoma, carcinoma, normal connective tissue.

Macroscopic Damage.

Animals.—Guinea-pigs of weight 500–700 g. were used. Some observations were also made on rats of weight 100–200 g.

Experiments.—The back of the animal was clipped with hair clippers and then shaved with an electric razor. The animal was anæsthetised with ether or nembutal (intra-peritoneal). The type of anæsthetic did not change the nature of the visible skin response. The burn was produced by means of the iron described in Section A of this paper. Two to four observations were made for each combination of temperature and burning time. The results were in good agreement, indicating that it mattered little what part of the back was exposed. Observations were made both on the immediate macroscopic damage, and on the later healing processes.

It is clear that with short applications of the burning iron, the temperature of the skin must be substantially below that of the iron; with longer applications, up to 6 minutes, the temperature may be expected to approach that of the iron, at any rate in the more superficial layer. Some information as to the maximum temperatures which can be withstood without irreversible damage will be obtained from rather longer applications, though for practical production of constant burns applications of 1 minute will be generally more convenient.

In preliminary observations, by R. A. Peters and R. W. Wakelin, reported to the Medical Research Council, it was found that temperatures over 50° C. are required to produce scab formation with 3-minute

applications. After exposure to 47° C. for 6 minutes, histological changes were not noticed by E. H. Leach. This shows that 47° C. is not dangerous, but that a rise of 3° C. above this may be. A typical development of scabs in 5 patches made at 55° C. is recorded in Table I.

TABLE I.

Guinea-pig burnt with 5 patches for 3 minutes, 55° C.   Sc. = Scab.

	6 hours after.			2nd day.			3rd day.			4th day.		
	1	2	3	1	2	3	1	2	3	1	2	3
Side of Animal R.	E.	E.	E.	E.	Sc.	E.	E.	Sc.	Sc.	Sc.	Sc. $\frac{2}{3}$	Sc. $\frac{1}{2}$
L.		E.	Slight Sc.		E.	Sc.		Sc.	Sc.		Sc. $\frac{2}{3}$	Sc. $\frac{1}{2}$

On the fourth day there was scab formation on all patches. Some formed earlier. 30 minutes at 70° C. and 10–20 minutes at 75° C. produced large scabs. It was found that the animals would tolerate 12 patches at 55° C. without death, though losses in weight were noticed; these are being made the subject of further investigation. Curiously enough these patches did not appear to be painful.

In the present work, which has amplified and extended these observations, 1 minute has been taken as standard. The number of animals burned with different combinations of temperature and burning time was too great to report all the results in detail. Two illustrative series of burns will, therefore, be described: a series of 1-minute burns with the temperature of the heating iron increasing from 45° C. to 80° C., and a second series with the burning iron at 55° C. for increasing lengths of time. Both series of burns were produced in guinea-pigs. Burns at other temperatures and for other burning times will be referred to only when it is necessary to demonstrate particular points. Similar observations were made with rats, and it was found that the results were very closely parallel to those of burns made on guinea-pigs.

Changes.—The immediate macroscopic changes readily observed were: erythema, flare, blanching, blueing, heat fixation, incipient blister formation, oedema and edge wheal. Observations on the later healing processes included the presence or absence of scab formation, and the rate of regeneration of epithelium.

Erythema.—This has been divided into three grades:

Erythema +   = erythema just visible.
Erythema + +   = erythema distinctly visible.
Erythema + + + = marked erythema.

Table II shows that an erythema was not seen until the temperature of the burning iron reached 50° C. This erythema was slow to develop (5 minutes), and was only transient (gone in 30 minutes). The erythema also increased with increase of burning time (Table III).

Erythema is less readily produced in a guinea-pig than in man. A 1-minute burn at 50° C. would produce a marked erythema ($+ + +$)

TABLE II.

Shows the rate of development and subsidence of signs after application of heat to skin of guinea-pigs. E. = erythema, Fl. = flare, Bl. = blanching. Œd. (Œdema) expressed as per cent. water content of tissue (average related to normal 100 per cent.) found 2 hours after application. Observations at each temperature made on 7 or more animals.

Heat applied for 1 minute. Temperature 45° –80° C.

Temp. ° C.	Time after application (minutes).					Œdema, per cent. normal, 120'.	Standard deviation.	Histological [3] damage, 120'.
	0'.	1'.	5', 10', 15'.	30'.	60'.			
45	Nil	Nil	Nil	Nil	Nil	103	± 27	A
50	Nil	Nil	E. +	Nil	Nil	97	± 9	A
55	Bl.	E. +	E. + + Fl. +	E. + Fl. +	E. +	119	± 28	D, E
60 [1]	Bl.	Bl.	E. + + Fl. + +	E. + + Fl. + +	E. + Fl. +	180	± 62	F
65 [1]	Bl.	Bl. Fl. +	Bl. Fl. + + +	Bl. . Fl. + +	Bl. Fl. + +	..	..	G or H
70 and 80	{Fl. + (Heat fixation) [2]	Fl. + +	Fl. + + +	Fl. + +	Fl. + +	(70) 199 (80) 202	± 18 ± 45	H

[1] At 60° C.–65° C. for 1 minute there was a separation down to the prickle cell layer, so that the epidermis could be wiped off.
[2] Heat coagulation to form a stiff yellow plaque.
[3] Three animals observed. See under "Microscopic Changes."

on human skin [Lewis and Love, 1926, and confirmed by us], while for the guinea-pig such a burn would produce but trifling erythema.

Flare.—An irregular reddening of the skin, spreading out from 1 to 5 mm. beyond the burned area, was constantly seen. The nature of this reaction has not been determined. We have called it a "flare," but it is not necessarily identical with the flare described in human skin by Lewis [1927] and thought by him to be caused by a local axon reflex. The reaction we have observed was possibly due to heat conduction from the burned area. There is evidence later that there is a large area of graded tissue damage beyond the edge of the heating iron.

Flare +   = Flare just visible.
Flare + +   = Flare distinctly visible.
Flare + + +   = Marked flare.

The greater the temperature of the heating iron, the more severe was the flare (Table II), but for longer exposures at 55° C. (Table III) the flare was not markedly increased.

Blanching.—A temporary blanching often formed before the development of the erythema, *e.g.* at 55° C. and 60° C., Table II. At these temperatures the blanching had completely disappeared and given way to redness by the end of 5 minutes.

Blueing.—After a 10-minute burn at 55° C. (Table III) the burned area was distinctly blue. This presumably indicated capillary stagnation as well as dilatation. Occasionally there was a blue patch (about 1 cm. in diameter) in the centre of the 5-minute burn at 55° C. The local erythema produced by 1-minute burns at all temperatures also showed a tendency to darken at the end of two hours.

TABLE III.

Shows the rate of development and subsidence after application of heat to skin of guinea-pigs. E. =erythema, Fl. =flare, Bl. =blanching. Œd. (Œdema) expressed as per cent. water content of tissue (average related to normal 100 per cent.) found 2 hours after application. Observations for each burning time made on 5 or more animals.

Heat applied for different times at 55° C.

Time, min.	Time after application in minutes.					Œdema at 120'.	Histo- logical damage, 120'.
	0'.	1'.	5', 10', 15'.	30'.	60'.		
1	Bl.	E. +	E. + + Fl. +	E. + Fl. +	E. +	99	E
2	E. + + Fl. +	E. + + Fl. +	E. + + Fl. + +	E. + + Fl. + +	E. + + Fl. + +	118	E
5	E. + + + Fl. +	E. + + + Fl. + +	E. + + + Fl. + + +	E. + + + Fl. + +	E. + + + Fl. + +	150	F
10	Fl. + Blueing of skin.	Fl. +	Fl. + + +	Fl. + +	Fl. + +	151	F

Heat Fixation.—At 70° C. or 80° C. the tissue proteins became heat coagulated, giving the exposed area the appearance and feel of a stiff hard yellow plaque.

Incipient Blister.—Rats and guinea-pigs do not blister when burned. If, however, the burning iron was at 60° C. to 65° C. for 1 minute, there was a separation of the prickle cell layer which could be demonstrated histologically. There was no obvious bleb formation. If the skin was gently rubbed with a piece of moist cotton wool, the whole of the epidermis peeled off from the area immediately under the iron.

This left a raw area of punched-out appearance,[1] not unlike the exposed surface seen when the blisters of a human second-degree burn are pricked and trimmed. If the epithelium was not then wiped off, the animal was sure to scratch if off within the next 24 hours.

Œdema.—Records were kept of the visible and palpable œdema which occurred for all burns at temperatures greater than 60° C. and for 5-minute burns at 55° C. More accurate data were obtained by determining wet and dry weights. Table II shows that there is a slight œdema at 55° C. and that from 60° C. onwards the œdema is marked. There is less than one chance in twenty that the difference between the figures at 50° C. and 55° C. is fortuitous ($t = 2.13$; for $P = 0.05$, $t = 2.12$) and little over one chance in fifty of the difference between the figures at 55° C. and 60° C. being fortuitous ($t = 2.52$; for $P = 0.02$, $t = 2.58$). The difference between the figures at 60° C. and 70° C. is not significant ($t = 0.119$; for $P = 0.9$, $t = 0.129$). With the 55° C. burn the œdema did not appear until the burning time reached 5 minutes (Table III). All the figures given in Tables II and III were obtained by guillotining the animal two hours after the production of the burn. It was of interest to determine how quickly the œdema appeared. Table IV shows that with 1-minute burns at 55° C. œdema

TABLE IV.

Rate of development of œdema (expressed as percentage of normal water content) as measured by wet and dry weight determinations in skin of guinea-pigs, burned for 1 minute with burning iron at the stated temperature.

Time after burning (minutes).	Œdema (percentage of normal water content).	
	Burning iron at 55° C.	Burning iron at 60° C.
0	100	100
1	96	107
5	91	122
10	102	116
30	105	142
120	99	180
180	103	206
240	..	199

did not develop even after 3 hours, and that with similar burns at 60° C. some œdema was present even after 5 minutes; it took 3 hours, however, for the full œdema to develop, and this lasted from 48 to 72 hours.

Edge Wheal.—A distinct wheal around the edge of the burning iron could be seen when the burns were at the higher temperatures. This

[1] This appears to be somewhat analogous to the effects of steam described by Wilson.

was most marked for the 70° C. and 80° C. burns, but was seen for temperatures as low as 60° C. for 1 minute and 55° C. for 5 minutes. This wheal was greatest for temperatures at which the tissue immediately under the heating iron was heat coagulated. If the wheal is produced by fluid exudation, it seems probable that there is more exudation into the non-coagulated areas beyond the edge of the iron than into the coagulated tissues directly under the iron. It is interesting to note that the temperatures at which the edge wheal is observed were similar to those at which œdema was found.

Scab Formation.—As previously indicated, with burns of 45° C. to 50° C. there was no scab formation, and this was true no matter how

TABLE V.

The late macroscopic changes in the skin of guinea-pigs burned for 1 minute at different temperatures. Each figure the mean of seven or more observations.

Temperature of burning iron.	Macroscopic appearance before scab formation.	Day scab formed ($\pm$ S.D.).	Day scab shed ($\pm$ S.D.).	Day of complete regeneration of epithelium ($\pm$ S.D.).
45° C.	No scab formation			
50° C.	No scab formation			
55° C.	Erythema	8 ($\pm$ 2)	14 ($\pm$ 4)	19 ($\pm$ 5)
60° C.	Erythema	2 ($\pm$ 1)	13 ($\pm$ 1)	25 ($\pm$ 3)
65° C.	Erythema	3 ($\pm$ 1)	16 ($\pm$ 2)	28 ($\pm$ 3)
70° C.	Yellow with flare	6 ($\pm$ 3)	17 ($\pm$ 4)	34 ($\pm$ 7)
80° C.	Yellow with flare	7 ($\pm$ 3)	20 ($\pm$ 5)	50 ($\pm$ 7)

long the burning iron was left in position. One-minute burns at 55° C. practically always produced a scab, so that the critical temperature for scabbing must be in the region of 50° C.–55° C. Tables V and VI show that the usual time for the scab to form was 6 to 8 days. This was true for all burns except those for 1 minute at 60° C.–65° C., and for those of longer duration than 2 minutes at 55° C. With these burns, the time for scab formation was much less. This was probably due to the presence in them of incipient blister formation.

At the lower temperatures, the burned skin had an erythematous appearance until the scab formed. With the burns produced by higher temperatures, the burned skin was yellowish and surrounded by a distinct red flare. The scab was always present for a period of one to two weeks before being shed.

Rate of Healing.—When the scab came off it revealed either a re-epithelialized area or a clean granulating surface which gradually became covered with epithelium. Usually, the outer portion of the burn was covered with new epithelium and there was a much smaller

TABLE VI.

The late macroscopic changes in the skin of guinea-pigs burned with the heating iron at 55° C. for different periods of time.   Each figure the mean of five or more observations.

Burning time (minutes).	Macroscopic appearance before scab formation.	Day scab formed ($\pm$ S.D.).	Day scab shed ($\pm$ S.D.).	Day of complete regeneration of epithelium ($\pm$ S.D.).
1	Erythema	8 ($\pm$ 2)	14 ($\pm$ 4)	19 ($\pm$ 5)
2	Erythema	8 ($\pm$ 1)	17 ($\pm$ 3)	28 ($\pm$ 5)
5	Erythema	4 ($\pm$ 1)	17 ($\pm$ 3)	37 ($\pm$ 5)
10	Yellow with flare	5 ($\pm$ 1)	19 ($\pm$ 6)	41 ($\pm$ 1)

granulating area in the centre.   This area became less and less and it is seen (Table V) that the time taken for the wound to be completely covered with epithelium increased with increase in temperature of the burning iron.   Similarly, with the iron at 55° C. the time taken for complete epithelial regeneration increased with the length of the burning time.

Microscopic Changes.—The pieces of skin were laid on shiny paper to keep them flat during fixation, which was done for at least 24 hours in formol saline.   A portion from the centre of the burn to the edge, with an equal portion of contiguous normal skin, was cut out.   After the paper was removed, the skin was dehydrated, cleared in cedarwood oil and embedded in paraffin.   Sections were cut at 8μ.   The standard staining method was Weigert's iron hæmatoxylin and eosin.   In addition, the following techniques were used on a selection of the specimens:—

1. Weigert's iron hæmatoxylin and Van Gieson.
2. Feulgen nucleal reaction.
3. Masson trichrome.
4. Orcein.
5. Pyronin-methyl green.

I. *General.*—The observations have been mostly restricted to the early phases of change due to burns.   These have not been previously described.   The necrosis does not differ from that caused by other types of trauma, such as bacterial; but here it has been possible to grade the trauma both in severity and duration, and to correlate the histological changes with macroscopic and biochemical changes.

To simplify tabulation of results, the changes have been artificially graded into eight Stages and lettered A to H for increasing severity.

Stage A.   Nuclei of epithelium swell, diffuse basophil staining of cyto-

plasm diminishes. Infiltration of polymorphs and pseudo-eosinophils into deeper layers of dermis. Reversible (see fig. 4).

Stage B. Nuclei now collapse leaving a perinuclear vacuole, but remain round. Further loss of basophil staining of the cytoplasm. Probably reversible (see fig. 5).

Stage C. Nuclei collapse further, become crescentic or flattened, small spaces appear between prickle cells. Possibly reversible (see fig. 6).

Stage D. Nuclei stain densely and uniformly—pyknosis. Some large spaces now formed between the prickle cells and the basement membrane contain a coagulum and basophil granules. Irreversible (see fig. 7).

Stage E. Nuclei crenated and fragmentary—karyorrhexis. Irreversible (see fig. 8).

Stage F. Partial heat coagulation of epithelial cells, which are preserved in a condition not very dissimilar from that of the normal cells. Irreversible (see fig. 9).

Stage G. Heat coagulation of epithelium with gross distortion of epithelial cells and their nuclei. Damage to collagen fibres of dermis. Irreversible (see fig. 10).

Stage H. As for Stage G but with marked damage to collagen fibres of dermis. Irreversible (see fig. 11).

From Table II, it will be seen that higher temperatures cause more marked effects. Two critical temperatures should be noted, as for the macroscopic changes. At about 50° C. there is a sudden onset of gross cellular damage. At about 60° C. a temperature is reached which causes coagulation of the surface epithelium and prevents cellular disintegration.

From Table III, it will be seen that application for longer times causes slightly greater effects, but that it is impossible to simulate the effects of much higher temperatures merely by prolonging the time of application. (This is probably due to the rapid damage of the thin layer concerned.)

From Table VII, it will be seen that the effect of the application of a temperature of 55° C. is very quick indeed in onset. Even when the skin was fixed in formol saline as soon as possible after burning, very definite changes had started. The process of cellular disintegration proceeded without further traumatic stimulation. The damage reached a peak at 30 minutes, although further necrosis with scab formation occurs in the succeeding days. With a more severe burn at 60° C. the time course is different. There is immediate heat coagulation of the epithelium and no great change occurs during the succeeding 2 hours.

Especially with the low temperatures, the heating of the epithelium appeared to have been slightly uneven, so when a given temperature

is said to cause a change of a certain stage, this means that the greater part of the burnt area shows this stage.

No special observations were made on the effects of mechanical trauma, but at the place where the normal skin was injured by the razor used for excision the epithelium showed changes grading from Stage C to Stage A. Sometimes large parts of the epithelium of the normal skin showed a mild Stage A change; this was probably due to trauma of shaving.

II. *Nuclear Changes.*—The first effect of trauma (Stage A) is a swelling of the nucleus, which instead of being flattened is now round. In optical section the area of the nuclei is increased about 50 per cent. (see fig. 4).

In Stage B the nuclei start to collapse or contract, the chromatin becoming dispersed and thereby causing a more uniform staining of the nuclei. A perinuclear space is left (see fig. 5). This space, as well as the various changes in the nuclei, may be a fixation artefact. These artefacts, if artefacts they be, represent true changes of some sort in the physicochemical state of the cells.

Further collapse (pyknosis) occurs in Stages C and D, and the nuclei become crescentic or flattened, leaving a large perinuclear space. Apparently at this stage nucleoprotein is escaping from them (see figs. 6 and 7). Granules of Feulgen positive material are found in the spaces between the prickle cells and in the incipient blister spaces separating them from the basement membrane. These granules stain in the same manner as nuclei with a variety of nuclear stains, such as hæmatoxylin, polychrome methylene blue, neutral red and carmalum. There can be no doubt that they are of nucleoprotein nature.

Slightly higher temperatures cause less collapse—possibly due to a partial heat coagulation (Stage E), but now the nuclei have a crenated edge or show obvious fragmentation—karyorrhexis (see fig. 8). The number of granules in the nucleoprotein material in the intercellular spaces increases. After 24 hours these may also be identified in the dermis to a depth of $\frac{3}{4}$ mm. By this time the nuclei have lost all their nucleoprotein, apparently by solution—karyolysis. They stain as faint acidophil structures having no basophil affinity and giving a negative Feulgen reaction. After 48 hours the number of the free nucleoprotein granules has diminished, and by the 72nd hour they have disappeared.

Higher temperatures (Stage F) apparently cause coagulation of the nucleoprotein, which, with temperatures of about 60° C., is preserved in a condition not dissimilar to that produced by formol fixation. The chromatin is diffuse instead of being present in granules (see fig. 9). Still higher temperatures cause coagulation with considerable flattening and distortion of the nuclei (see figs. 10 and 11).

Stages G and H. A similar but less marked series of changes can

be seen in the nuclei of the hair follicle cells. Nuclei of connective-tissue cells become pyknotic.

III. *Cytoplasmic Changes.*—In 'Stages A to E there is an apparent increase in the volume of the cytoplasm. In Stage F the volume is normal, and in Stages G and H it is greatly reduced.

In Stage C the cells, especially the prickle cells, become separated from one another and from the basement membrane to which they are attached by thin processes (fig. 6). In Stages D and E this change goes further and many of the prickle cells virtually disintegrate. In sections stained with hæmatoxylin, the diffuse basophil staining of the cytoplasm is seen to be diminished in Stage A and to have disappeared in Stage C. 'But in Stages F–H there is less loss. If sections are stained with pyronin-methyl green, the normal epithelial cells show brilliant red cytoplasmic granules. In Stage B, and even more in Stages C–E, brilliant red granules and a coagulum of the same colour can be seen between some of the prickle cells, and between them and the basement membrane. At the end of 24 hours all signs of this substance in the incipient blisters has disappeared. In Stage H the cytoplasm stains bright red with pyronin-methyl green, indicating that here the basophilic substance has not escaped. The keratohyalin granules of the stratum granulosum tend to swell, become more widely separated and fewer in number in Stages B to E. In Stages F to H they are quite normal.

TABLE VII.

Stage of histological change caused by a burn of 1 minute duration at 55° C. and 60° C. Skin removed at different intervals after burning.

	½ min.	1 min.	5 mins.	10 mins.	30 mins.	120 mins.
At 55° C.	B	C	B–C	B–C	D	D
60° C.	F	F	F	F	F	F

IV. *Collagen Fibre Changes.*—In Stages A to E the collagen fibres appear to be quite normal. In Stage G they are swollen and the component fibrils partly separated. The fibres have a decreased affinity for acidic dyes such as eosin and acid fuchsin. Their affinity for basic dyes, such as hæmatoxylin, safranin and orcein, is increased. In Stage F these changes are less marked and in Stage H much more marked.

V. *Œdema.*—This was not gross enough to be obvious in the sections to visual judgment except in and around the layer of striated muscle of the skin. Measurement of the thickness of the dermis gave some indication. The variation in the thickness of the normal skin was too great to allow it to be a method of estimating œdema.

In Stage H burns a few of the interfibrillar spaces are filled by a basophil coagulum of unknown nature.

Œdema of the striated muscle of the skin, which lies about 1·5 mm. from the epithelium, occurs in Stages F–H burns. In Stage F burns it is present only in a small part of the centre of the burn, not closer than 5 mm. from the edge. In Stage H burns it spreads over a wider area and is visible 1·0 mm. from the edge.

VI. *Incipient Blister Formation.*—Blisters formed are never large. The largest seen was 0·5 mm. in diameter. Care has to be exercised to differentiate artefact separation of the epithelium from true blisters. Only when a coagulum can be seen filling the gap between the epithelium and the basement membrane can the space be identified for certain as a blister.

The process of blister formation starts with separation of the prickle cells from one another. They show great adherence to the basement membrane, and long drawn-out processes often keep the epithelium tied to it. At this stage, Stage C granules and a coagulum staining bright red with pyronin can be seen in many of the spaces. This has been seen 1 minute after burning.

In Stage D, some of the processes of the prickle cells have disintegrated, allowing a large gap to form between the epithelial cells and the basement membrane (see fig. 7). Granules of nucleoprotein make their appearance now, both in the blister spaces and in gaps between prickle cells. Their appearance definitely succeeds that of the pyronin staining substance, which is apparently derived from the basophil granules of the cytoplasm. Although some of the blisters now contain a coagulum staining with pyronin, most contain a slightly basophil coagulum which does not stain with pyronin and does not give the Feulgen reaction for nucleoprotein.

It seems likely that blisters are formed by seepage of tissue fluid under pressure into spaces caused by the partial disintegration of the prickle cells.

This type of incipient blister differs from that described macroscopically, because it occurs at a lower temperature, about 55° C. instead of 65° C. At the latter temperature the epithelium apparently remains adherent to the connective tissue unless it is detached by mechanical trauma. The ease with which this occurs may probably be explained by the hypothesis that the epithelium has become hardened by heat coagulation and has lost its elasticity. It cannot here be said whether the human blister corresponds to one or both of these types.

VII. *Polymorph Infiltration.*—This is most marked in Stages D to F. In Stages A to C and G to H it is easily detectable. In all stages it extends into the unburnt area. In a stage D burn it extended 0·5 mm. and in a Stage H burn 1·5 mm. In Stages G and H more infiltration occurs in the deepest layers of the dermis and the underlying striated

muscle than in the region where the most marked damage to the collagen fibres has occurred.

In all cases there is much less infiltration into the superficial layers of the dermis containing the hair follicles (stratum papillare) than in the deeper stratum reticulare. This is true both for the burnt area and the adjoining unburnt skin. Both true polymorphs and pseudo-eosinophils infiltrate.

Horizontal Distribution of Changes.

There is no gradient of change in the burnt area except at the edge. In a Stage D burn epithelial changes extend over a distance of 1 mm., and in a Stage H burn about 2 mm. The ability to recognize the intermediate stages here in the appropriate order gives added assurance to the grouping adopted (see fig. 12). It also indicates a gradient of temperature either under, or just outside, the edge of the burning iron.

A similar gradient of change occurs in the collagen fibres. With a Stage H burn the gradient of changes extends over a distance of 1·5 mm.

Vertical Distribution of Changes.

In the central portion of the burn the depth to which changes occur depends on the severity of the epithelial changes. No appreciable difference exists between burns of a lower temperature of long duration and those of a higher temperature and shorter duration, provided that the changes in the superficial epithelium are the same. In the hair sheaths and follicles a gradient of cellular damage can be followed downwards. The changes are not so clear cut, but karyorrhexis has been observed in the hair sheath cells at a distance of $\frac{1}{2}$ mm. from the surface. A few examples of the distribution of the changes will illustrate this most important point.

	Surface.	0·12	0·25.	·37 mm.
1-minute burn at 55° C.	D	B	A	A
1-minute burn at 65° C.	F	E	C	A
10-minute burn at 55° C.	F	E	D	B

DISCUSSION.

1. An outstanding point of this work is the comparatively low temperature required to produce permanent or irreversible damage. The critical temperature is in the region of 50° C. for applications of the burning iron for three minutes; at 55° C. 1 minute is enough; and similar amounts of destruction are produced by 10 seconds at 75° C. Hence it can be understood how a very few seconds at higher temperatures cause damage. Burns at 55° C. for 1 minute produce very good scabs. All this is in general agreement with the earlier work of Cohnheim [1873] and McMaster and Hudack [1934] for burns of the

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loss of its nucleoprotein by karyorrhexis and karyolysis within twenty-four hours, and the cytoplasm loses its affinity for basic stains.

In the second type, temperatures of 60° C.–80° C. (Stages F–H) cause an apparent coagulation of the epithelium. There is less loss of nucleoprotein and basophil affinity of the cytoplasm. Collagen fibres show considerable damage with loss of acidophil affinity. At the same time there is a deeper zone in which some of the changes noted under A to E are also present. If all the burn was of the second type, we should say from histological evidence that there was nothing to support the idea of toxic substances leaving the skin. On the other hand, in any less heated area, we shall have conditions approximating to those of the first type. Here we have histological evidence that at least two substances come from the cells:

      (a) The pyronin staining substance.
      (b) The nucleoprotein.

We have therefore made some progress towards a histological proof of the hypothesis advanced in the introduction by showing that this partly damaged layer (see fig. 1) can liberate histologically detectable substances. It is certainly suggestive that these changes may occur quickly, some within a few minutes and all within 24 hours, which would agree in time with the toxæmia to which Wilson and colleagues have often referred, and which must be distinguished from bacterial toxæmia. Still, as yet we have no proof that the substances seen are toxic, and must not shut our eyes to the possibility that any such might be histologically undetectable. Either could arise as the result of altered tissue metabolism.

With these cautions in mind, it is tempting to speculate upon correlations found between tissue reactions and the histological findings. The pyronin staining substance, for instance, is the first to be liberated (i.e. at the lowest temperatures) and corresponds in time with the liberation of the H substance [Lewis, 1927] or leucotoxin [Menkin, 1940] and the appearance of leucocytic infiltration and erythema.

The œdema could be related either to the migration of nucleoprotein or to the change in structure and staining properties of the collagen fibres.

3. This work forms a basis for the correlation of macroscopic, microscopic, and biochemical data. We can put some of these together already, for temperatures of 55° C. (1 minute exposure).

There is immediate blanching followed by the following:—

        After 30 mins. Erythema.
                 Flare.
                 Slight œdema.
      After 8 days   Scab formation.

ear. Skin temperatures must be below that of the burning iron, and may prove sufficiently low to fall into line with the published reports of the temperature of inactivation of transplants and tissue cultures. Work is now in progress to verify these points.

The erythema of 55° C. and 60° C. (1 minute) burns is preceded by a transient blanching, which conforms with the momentary cessation of the circulation described by McMaster and Hudack.   Burns at 50° C. merely give an erythema, and the histological damage is reversible. The lower temperature of 47° C., even if applied for many minutes, produces no macroscopic change, though there may be reversible microscopic effects. As this should give temperatures in the skin more nearly approximating to that of the iron, there must be a sharply critical point in the biochemical constituent concerned in the cells. Preliminary observations upon the thermostability of a few respiratory enzymes have been made already by Dr. B. Olafson; some have also been made upon the skin enzymes; and they will be reported in due course.

The œdema found after exposure to temperatures of 60° C.–70° C. results in a large increase in water content of the skin, which would amount to as much as 57 per cent. of the blood volume if half the body surface were burnt.   This represents loss of circulating fluid to the skin alone and excludes loss to subcutaneous tissue, muscle, etc., and the large external loss by exudation from the raw surface This finding, like those of Underhill and his colleagues [1930], suggests that the loss of fluid alone may be responsible for large changes in metabolism.

The calculation for the increase in water content of the skin is as follows:—

500 g. Guinea-pig.

Weight of skin (excluding head and feet) = 63 g.

Water content of normal skin   = 70 per cent.,

i.e. 44 g. water.

Increase of water after burning is 100 per cent. of normal, *i.e.* 22 g. if half body surface burned.

Blood volume   = 7·7 per cent. of weight

= 38·5 g.

∴ Fluid loss to skin if half surface burned $= \dfrac{22}{38\cdot5} \times 100$

= 57 per cent. of blood volume.

This excludes fluid loss to subcutaneous tissue, muscle, etc.

2. Two main types of histological changes due to exposure to temperatures of 45° C.–80° C. are found.   In the first 50° C.–60° C. (Stages A–E) there is found a considerable degree of cellular destruction in the epithelium; the nucleus undergoes a change resulting in complete

7. The observations recorded here are readily reproducible and provide a standard method of burning for experimental work.

III. *Microscopic Changes.*

1. The histological effects of mild burns of varying intensity and duration have been described in detail. They follow the general picture previously recorded in necrotic changes.

2. Two different types of reaction are described; with milder burns there is cellular disintegration; with more intense burns, heat coagulation.

3. In more intense burns there is a peripheral shell of changes characteristic of burns of lower temperature.

4. Two substances are lost by the epithelium of skin which has been subjected to a burn:

(*a*) Basophil granules from the cytoplasm.

(*b*) Nucleoprotein from the nuclei.

These two substances can be identified in the intercellular blister spaces.

5. Collagen fibres change in structure and staining affinity in the more intense burns.

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This irreversible change shows loss of pyronin staining material, followed by loss of nucleoprotein; only the latter seems to be associated in time with the incipient formation of blister-like gaps. It should be stressed that E + for the animal is the equivalent of rather severe reaction in man.

Some theoretical points are also worth noting. The earliest change, a reversible one, is swelling of the nuclei, the explanation of which, when it comes to hand, may prove of great interest. Is it merely due to the increased metabolic rate or does some shift in the pH balance between the nucleus and cytoplasm take place? There is known to be normally a greater alkalinity in the nucleus as compared with the cytoplasm.

In large burns it is obvious that there is likely to be a larger area of moderately heated tissue; and it would be expected that the larger the area, the greater would be the toxic effects; these would be therefore worse in the large burn of moderate temperature.

SUMMARY.

I. With the object of producing standard low-temperature burns in animals, and of studying the area of tissue only partly damaged in a burn, a burning iron has been made capable of applying temperatures from 45°–80° C. to the skin; with this the amount of heat and temperature causing skin damage has been studied, and the macroscopic and microscopic damage due to graded temperatures have been delineated.

II. *Macroscopic Changes.*

1. Graded temperatures of 45°–80° C. have been applied to the skin of shaved, anæsthetised guinea-pigs, and in some cases rats, for times varying from 10 sec. to 6 and 10 min. Observations have been made of the development of erythema, flare, blanching, blueing, heat fixation, incipient blister formation, œdema, and edge wheal, as also upon the later scab formation and rate of epithelium regeneration.

2. Applications of 47° C. up to 6 minutes produce no visible change.

3. At 50°–55° C. applied for 1 minute and over, there is a critical temperature for the development of permanent and irreversible damage; in animals good scab formation occurs after burning at this temperature.

4. After temperatures of 60°–65° C. the epidermis can be peeled off from the exposed area, leaving a punched-out exposed surface area somewhat like the exposed human blister.

5. A temperature of 70°–80° C. for 10–20 seconds produces severe scabbing.

6. Observations of œdema formation have been checked by wet and dry weights of specimens of skin taken 2 hours after heating. At 55° C. there is some change, but this is more definite at 60° C.

3.—Normal skin of guinea-pig stained with t's iron hæmatoxylin and eosin. Magnifica- 170. Same stain and magnification in figs. 4–11.

FIG. 4.—Stage A. 50° C. for 1 minu Removed 2 hours later.

FIG. 5.—Stage B. 55° C. for 1 minute. Removed 5 minutes later.

FIG. 6.—Stage C. 55° C. for 1 minu Removed 1 minute later.

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FIG. 4.—Stage A.   50° C. for 1 minute
Removed 2 hours later.

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Temperature Burn."
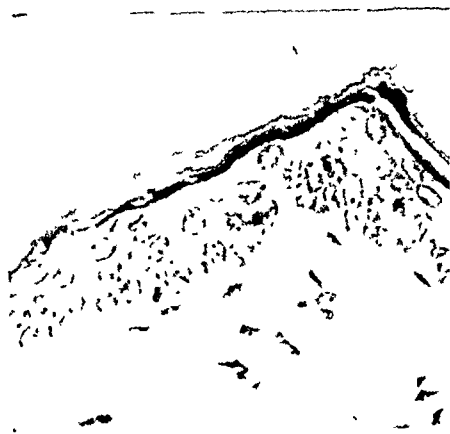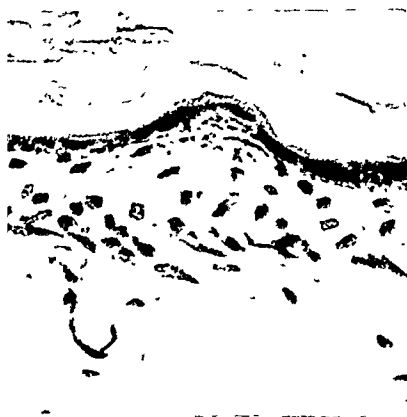
7.—Stage D.   55° C. for 1 minute.
Removed 2 hours later.

FIG. 8.—Stage E.   55° C. for 1 minute
Removed 2 hours later.

FIG. 9.—Stage F.   60° C. for 1 minute.
Removed immediately.

FIG. 10.—Stage G.   70° C. for 1 minute.
Removed 2 hours later.

NOTE ON THE SPINAL ORIGIN OF VASOCONSTRICTOR FIBRES TO THE ARM IN MAN. By J. R. LEARMONTH and R. L. RICHARDS.* From the Department of Surgery, University of Edinburgh.

RECENTLY a clinical problem has allowed us to make certain observations on the reflex vasomotor control of the arm.

The patient was an intelligent youth of nineteen, who had been referred to J. R. L. by the courtesy of Mr. W. A. Cochrane. He suffered from spastic right hemiplegia dating from birth. This was complicated by gross athetosis of the right arm and right shoulder girdle, which was a serious hindrance to his getting about (he had to hold the right arm with his left hand) and a source of great mental distress to him. The lad and his parents were very anxious that the movements should be stopped. The only procedures worth considering were forequarter amputation, or intraspinal division of the anterior roots destined for the brachial plexus. After full discussion the parents chose the latter procedure, and on 12th October 1942 the anterior roots on the right side were divided, within the dura, from the *fifth* cervical to the *second thoracic* segments, inclusive.

After operation the forequarter was completely paralysed, with the possible exception of the rhomboids. It was not easy to be sure of this, because the shoulder girdle was still moved involuntarily, but much less violently, by the trapezius. Sensation was unimpaired save for some hypæsthesia in the second thoracic segment.

Vasomotor Responses.—On 25th November 1942 the patient was placed in a cool room. Thermocouples were attached to the left middle finger (control), and to the right little finger, right middle finger, and right thumb, and readings of skin temperature were taken at intervals of 3 minutes (fig. 1). At 23 minutes the feet and legs were immersed in water at 45° C. At 39 minutes the skin temperatures of the fingers began to rise, the rate of rise being nearly the same on both sides; the temperatures reached were somewhat higher on the left side. At 60 minutes the following temperatures were recorded on the right (paralysed) side: thumb 32° C., index 34° C., middle

34·5° C., ring 34° C., little 34° C.   It was noted that the ulnar side of the right hand felt warmer than the radial side; the thermocouples
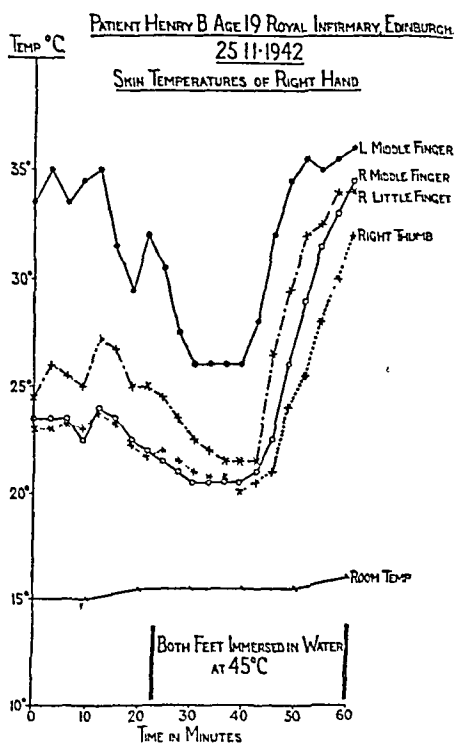
FIG. 1.

were transferred to the thenar and hypothenar eminences, and to the wrists.   The clinical impression was confirmed by the following readings:—

					Right.	Left.
Thenar eminence	.	.	.		23·5° C.	32° C.
Hypothenar eminence		.	.		34° C.	34·5° C.
Wrist	.	.	.	.	24·5° C.	31° C.

SUMMARY.

The observations show that, in this patient, reflex vasomotor response in an arm was normal, when the most cranial source of its preganglionic vasoconstrictor fibres was the *third thoracic* segment of the spinal cord.

RENAL DAMAGE DUE TO CRUSH INJURY AND ISCHÆMIA OF THE LIMBS OF THE ANÆSTHETIZED DOG.

By M. GRACE EGGLETON, K. C. RICHARDSON, H. O. SCHILD, and F. R. WINTON. From the Department of Pharmacology, University College, London.

AIR-RAID casualties suffering from extensive and prolonged crushing of limbs often die a week or so later from renal failure. This renal condition in man has been described by Minami [1923], Beall, Bywaters, Belsey, and Miles [1941], Bywaters and Beall [1941], Mayon-White and Solandt [1941], and others. At the suggestion of Dr. J. M. McMichael we undertook an experimental study of the condition, and in the first instance chose an experimental preparation, the anæsthetized dog, on which the analysis of renal function has been relatively fully developed. If prolonged crushing and ischæmia of limbs could produce a rapid failure of renal function under conditions of an acute experiment, the affair would become more accessible to experimental analysis, both in respect of the mechanism of the lesion and of the testing of various therapeutic procedures.

Experiments conducted on these lines showed marked renal damage as indicated by the first nine sets of observations summarized in Table I, A. The hypothesis was then explored that a toxic agent released from the injured limbs and transmitted through the blood-stream to the kidney, passed through the glomerulus and was concentrated in the tubule, exerting there its maximum effect. If this were so, one might expect that a period of low urine flow accompanying the fall in arterial pressure, as in human cases, would afford conditions for maximum damage to the kidney. Fluids or diuretics were, therefore, administered to counteract the anuria in the hope of diluting the toxic agent in the tubules and reducing the damage. A second series of eight sets of observations were made on dogs in which a brisk urine flow was maintained throughout by intravenous infusion of various diuretics, but as indicated in Table I, B the renal damage was no less severe than in the dogs of the first series.

A more crucial test of the hypothesis that the toxic agent acted in virtue of its concentration in the tubules was provided by comparison of two kidneys in the same dog, one of which was submitted to increased

ureter pressure, so reducing the urine flow and increasing the concentration of those substances which, like creatinine, are concentrated by the kidney. These sets of observations on five dogs (Table II) justified a final rejection of this hypothesis.

An analysis of the variation of creatinine and chloride excretion, of the pigment in the urine, and of the post-mortem and histological appearances of the kidneys in these experiments, together with a few additional experiments, for example on the influence on renal function of low arterial pressure due to intravenous infusion of histamine without crushing of limbs, has led to provisional conclusions concerning the nature of the lesion which are formulated below.

METHODS.

Most of the dogs in this series were anæsthetized with nembutal (Abbott, 0·7 c.c. per kg. body-weight) injected intraperitoneally, with supplementary doses of the same drug usually administered slowly intravenously later in the day as required. A few of the dogs were anæsthetized with ether-chloroform mixture before intravenous injection of chloralose (0·1 g. per kg.) and were found to react in the same way as those anæsthetized with nembutal.

Creatinine, usually about 6 c.c. per kg. of 5 per cent. solution, was injected intravenously. The ureters were cannulated, and the urine from each collected in a 5-c.c. graduated tube and its rate of flow continuously recorded [Winton, 1939]. The carotid arterial pressure was also recorded. Blood samples were collected from the brachial artery. A continuous intravenous infusion of a solution of creatinine and urea was maintained at a rate estimated to replace the loss of these substances in the urine.

Creatinine was estimated as described by Eggleton, Pappenheimer, and Winton [1940 a] and chloride by Volhard's method.

Since the characteristic conditions which produced renal damage in man appeared to involve both damage to limb muscles and prolonged ischæmia, we reproduced these conditions in the dog by binding rubber tubing fairly tightly round both hind limbs, from ankle to thigh. Soon after applying the bandage, each thigh was crushed in a large vice for about five minutes, some of the muscle being further injured by hitting with a hammer. The bandages were left in position for 4–5 hours, so as to produce ischæmia corresponding with the prolonged compression of the limbs in air-raid casualties.

Histological Methods.—Routine histological examination of the kidneys was made from paraffin sections of material fixed in the Dubosc-Brasil modification of Bouin's fluid (alcohol–picro–formol) and stained with Masson's hæmatoxylin, light green, eosin. *The mitochondria of the cortical tubule epithelium were fixed by Schridde's method and*

stained with Heidenhain's iron hæmatoxylin.   A few tests were made for the presence of hæmoglobin or myohæmoglobin in frozen sections of material fixed according to the method of Slonimski-Lapinski and stained with Lison's zinc-leuco prepared from acid violet.

RESULTS.

A typical experiment showing the influence of crushing and prolonged ischæmia of both hind limbs is depicted in fig. 1.   During the

FIG. 1.—Anæsthetized dog (9·5 kg.), showing changes in renal function, (a) when circulation is readmitted to crushed and ischæmic limbs, (b), when the blood volume is restored, and (c) when the urine flow is increased by a diuretic.

period of ischæmia, usually in the latter half of the period, the arterial pressure rises, the urine flow falls, and there is a small but definite fall in creatinine clearance.   During the same period the chloride concentration in the urine falls.   These changes could be explained by an ingravescent change in sympathetic tone reducing glomerular capillary pressure.   At the time marked "release," the bandages were removed from the limbs, and the doughy consistency of the thighs

was transformed into the normal more resilient one in a few minutes by massage. Œdema developed in the limbs, was readily detected half an hour after the release, and progressed thereafter, becoming severe in degree.

In the experiment described in fig. 1, and in similar experiments forming the first group in Table I, the fall of arterial pressure which followed immediately on releasing the limbs was not counteracted for one hour, the urine flow meanwhile remaining at a very low value or ceasing completely. Restoration of the arterial pressure to a value which produced a urine flow of the same order as before release of circulation to the limb, resulted in a creatinine clearance averaging one-third of the pre-release value.

TABLE I.—CHANGES IN CREATININE CLEARANCE (SINGLE KIDNEY) FOLLOWING INJURY TO LIMBS.

Expt. No.	Wgt. of dog, Kg.	Creatinine clearance, c.c./min.			Urine flow, c.c./min.		Blood pressure, mm./Hg.		Pigment in urine.
		Before.	After.	Per cent. dec.	Before.	After.	Before.	After.	
A. Release of Limbs followed by Period of Anuria lasting 45–65 Minutes.									
2 *	15	22	1·7	93	0·15	0·08	195	130	+
4 *	6	11·8	12·8	..	0·94	1·50	195	140	0
5	11	29	12	59	0·17	0·40	135	100	0
6 a *	9·5	29	4·4	85	0·11	0·11	105	95	+
6 b *	9·5	21	3·8	82	0·10	0·17	105	95	+
7 *	11·5	22	11	50	0·07	0·06	125	90	0
9 †	11	28	4·2	85	0·08	0·18	125	145	+ +
23 a	10	32·5	14·1	56	1·14	0·25	155	105	+
23 b	10	34	15·1	56	1·22	0·25	155	105	+
B. Anuria prevented by Intravenous Diuretics.									
10 *	13	11·5	5·4	53	0·25	0·59	160	90	+
11 a	11·5	22	10	55	0·36	0·26	130	100	+ +
11 b	..	22	13	59	0·35	0·34	..	..	+
12	9	14·7	3·7	75	0·40	0·39	115	97	0
15	23	48	13	73	0·13	0·39	110	105	+ + +
16	15	19·4	5	74	0·35	0·30	120	100	+
17	12	22·5	6·3	72	0·58	0·33	115	105	+
18	12·5	30·2	8·3	73	0·51	0·78	100	60	+

* Control sample taken before period of binding; elsewhere before binding.
† Limbs ischæmic but not crushed.

The Effects of Diuretics on the Damaged Kidneys.

It will be seen from fig. 1 that intravenous injection of a large dose of hypertonic saline (80 c.c. of 20 per cent. NaCl) produces not only a 10-fold increase in urine flow, but more than doubles the creatinine

clearance, restoring it to about one-half of its original value. The average values for observations on four damaged kidneys were that a dose of sodium chloride of 1·4 g./kg. injected in 20 per cent. solution increased the urine flow 15-fold, and the creatinine clearance just over 2-fold. In similar observations on eight damaged kidneys, 5 per cent. sodium bicarbonate in doses averaging 0·45 g./kg. more than doubled the urine flow and increased the creatinine clearance by 30 per cent. When saline, gum saline, or ox serum was injected into dogs with damaged kidneys in amounts averaging (in five observations) 32 c.c./kg., in circumstances in which there was no large rise in arterial pressure, the urine flow increased on the average 7-fold, and the creatinine clearance 2·2-fold. Most of our observations on the action of sodium sulphate are not comparable with the above, as a 10 per cent. solution was commonly infused slowly over long periods, this being a reliable method for maintaining a brisk urine flow from the damaged kidneys. A single dose averaging 0·9 g./kg. sodium sulphate was administered rapidly in only two animals, the mean increase in urine flow being 7½-fold and that in creatinine clearance being 40 per cent.

It is difficult to know on what basis to compare the merits of these diuretic procedures. The increase in the absolute value of the creatinine clearance described above is probably a good indicator of the change in excretory capacity of the kidney. It may be recalled, however, that the increase in urea clearance is proportionately greater than that in the creatinine clearance by an amount which depends on the increase in urine flow [Eggleton, Pappenheimer, and Winton, 1940 b]. Another basis of comparison is obtained by calculating the increase in creatinine clearance corresponding with a doubling of urine flow—assuming that over the relevant range the increases in these two variables are proportional. In the above observations, the increase in clearance corresponding with 100 per cent. increase in urine flow was 26 per cent. with sodium bicarbonate, 7 per cent. with hypertonic saline, and 20 per cent. with increase in blood volume (saline, gum saline, serum). These values may be contrasted with those obtained by Eggleton et al. [1940 b] on a similar preparation with undamaged kidneys; in these a rise in arterial pressure which doubled the urine flow raised the clearance by 14 per cent., injection of Ringer's fluid was accompanied by practically no change in the clearance, and urea diuresis actually involved a 10 per cent. reduction in creatinine clearance. If, however, urea be administered to a dog with abnormally low urine flow due, for example, to low arterial pressure, it will increase the creatinine clearance [Winton, 1934 b], and this phenomenon may account for the usually large increases in clearance produced by diuretics in the damaged kidneys.

Renal Function when Anuria is Prevented.

The problem arises whether the condition of anuria persisting for a considerable period after readmission of circulation to the limbs, as in experiments summarized in Table I, A, is an essential antecedent of the renal damage which subsequently becomes manifest. Stasis in the tubules might have deleterious consequences for a number of reasons, for example, the high concentration which a diffusible poison might thus attain, or the long time available for a substance slowly precipitated to aggregate and become capable of blocking the tubules.

In all but one experiment, it was found relatively easy to prevent this anuria and to maintain the urine flow at about its previous level by adjusting to a suitable rate an intravenous infusion of a diuretic, usually 10 per cent. sodium sulphate. It will be seen from Table I, B, which summarizes such experiments, that the damage to the kidney was of the same order as that in the experiments (Table I, A) in which there was an anuric period.

Evidence from Partially Obstructed Kidneys.

It has been argued [Winton 1934 *b*] that an increased creatinine clearance produced by reduction in reabsorption of water by a tubular diuretic can be most simply explained by supposing that the tubules are not impermeable to creatinine, some of which is restored to the blood stream by passive diffusion. The beneficial effect of diuretics on the damaged kidneys described above might also be explained in this fashion, but in this instance an additional factor might be predominant if the damage were due to a filterable poison which became concentrated in the tubules and exerted its action in virtue of its concentration at this site. Diuresis would involve a low concentration of poison as it does of all "low threshold" substances and consequently less toxic effect. The latter hypothesis can be tested by observing the effects of partial blocking of urine flow by increased ureter pressure.

In the class of experiment represented by fig. 2 one kidney was partially obstructed before, or early in the ischæmic period, and kept so till some hours after the readmission of circulation to the limbs. Soon after this readmission the creatinine concentration in the urine from the obstructed kidney (fig. 2) was four times as high as that in the urine from the unobstructed kidney. If the poison had been similarly more concentrated on the obstructed side, the severity of the damage should have been greater on that side. In fact, the obstructed side, after removal of the obstruction, was, if anything, a little less severely damaged than the unobstructed side. Unless the effect of the poison is fully and immediately reversible, this excludes the hypothesis that the damage is due to a diffusible poison which is concentrated in the tubules and produces its effect on the tubular cells in

:ontact with it, and confirms in this respect the less specific indications
lerived from experiments considered in the previous section.

The observations depicted in fig. 2 were confirmed in other animals
is shown in Table II. It will be seen that when a damaged kidney
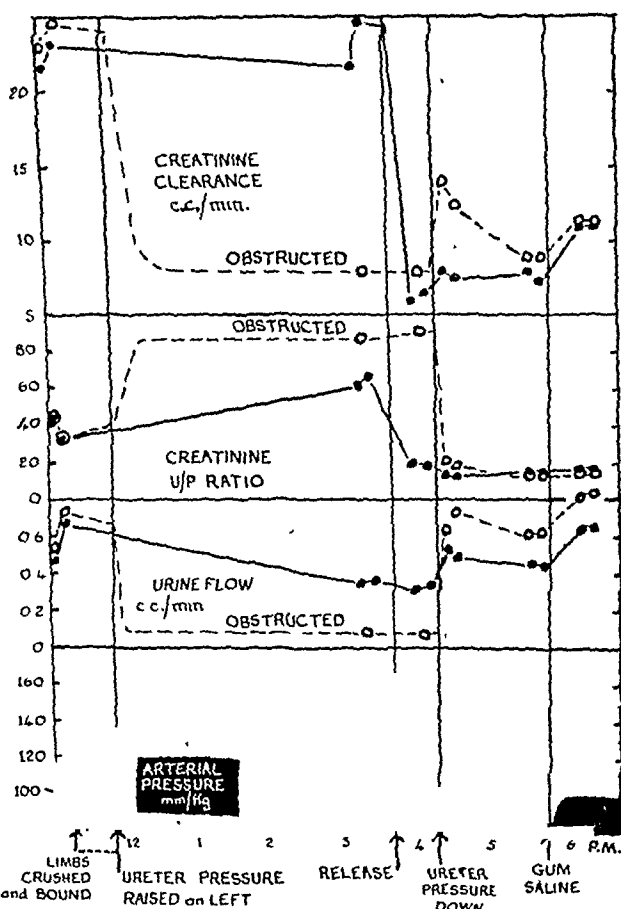
FIG. 2.—Anæsthetized dog (12 kg.), showing effects on renal function (a) of
crush and ischæmia, and (b) of readmission of circulation to the limbs, when one
kidney is partially obstructed by increase in ureter pressure. The effects of
removal of the obstruction show that the previously obstructed kidney is not more
damaged than the control kidney.

has been obstructed for some hours and is then released, its creatinine
clearance is usually rather higher than that of its fellow which has also
been damaged but not obstructed. This effect is small, but the more
significant since in control experiments with undamaged kidneys, a
long period of partial obstruction of one kidney results after its release
in a lower creatinine clearance on the previously obstructed side than
on the unaffected side, as shown in fig. 3. The urine flow from the

previously obstructed kidney is, however, greater than that of its un-
obstructed fellow, both in the presence and absence of damage, and it
seems that prolonged partial obstruction produces some fairly long
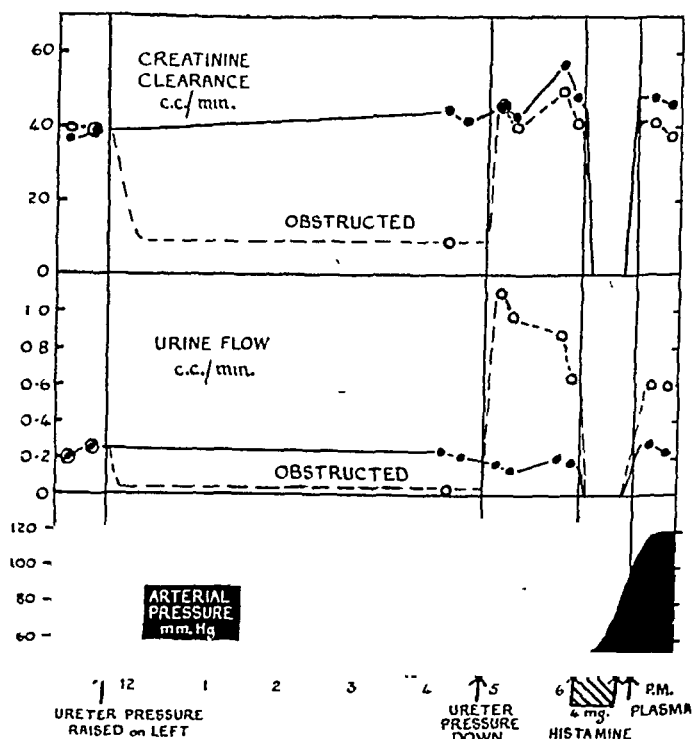
FIG. 3.—Anæsthetized dog (13 kg.), limbs undamaged, showing (a) the effects
of prolonged partial obstruction of one kidney, (b) the effects of removal of such
obstruction, and (c) the effects of a period of low arterial pressure due to intravenous
infusion of histamine—restoration of pressure showing unimpaired renal function.

lasting diminution in water reabsorption which does not occur after
partial obstruction lasting for an hour or less.

Changes in the Urine during Ischæmia of the Limbs.

During the period of 4–5 hours which involved as complete an arrest
of circulation in the hind limbs as a tightly bound rubber tube covering
much of the limb surface could achieve, there was usually a slow pro-
gressive rise in arterial pressure accompanied by a fall in creatinine
clearance (Table III). This may well be due to a progressive rise in
sympathetic tone, producing a fall in glomerular capillary pressure.
The associated fall in urine/plasma concentration ratio of chloride
(Table IV) is greater than might be expected on this hypothesis, but
the effect may have been augmented by the employment of diuretics

TABLE II.—THE INFLUENCE OF PARTIAL URETERAL OBSTRUCTION ON THE RENAL DAMAGE DUE TO CRUSHING AND BINDING OF LIMBS. (ONE KIDNEY WAS PARTIALLY OBSTRUCTED BY RAISING URETER PRESSURE, THE OTHER WAS LEFT UNOBSTRUCTED.)

A. Before releasing limbs: one ureter obstructed. Showing concentration of creatinine (higher U/P ratio) and diminution of clearance on obstructed side.
B. After releasing limbs: one ureter obstructed. Showing fall in clearance on both sides with relative concentration of creatinine (and of hypothetical toxin) on obstructed side.
C. After removing ureter obstruction. Showing on the side which had been obstructed clearances which are, if anything, slightly higher than in the controls.

Expt. No.	Wgt. of dog, Kg.	Ureter.	Urine flow, c.c./min.			Creatinine U/P ratio.			Creatinine clearance, c.c./min.			Blood pressure, mm./Hg.		
			A.	B.	C.	A.	B.	C.	A.	B.	C.	A.	B.	C.
12	9	Obstructed	0·06	0·043	0·38	100	42	14·4	6	2·1	5·5	115	95	115
		Unobstructed	0·4	0·4	0·51	37	9·4	12·0	14·7	3·7	6·1			
15	23	Obstructed	0·058	0·039	0·445	550	130	41	32	5·1	18	115	100	105
		Unobstructed	0·13	0·39	0·51	380	33	28	48	12·5	14			
16	15	Obstructed	0·12	0·076	0·6	81·5	24·5	12·7	9·8	1·9	7·7	125	100	95
		Unobstructed	0·35	0·3	0·3	58	16·6	12·6	19·4	5	3·8			
17	12	Obstructed	0·088	0·086	0·62	88	92	14·4	8	8	9	165	105	85
		Unobstructed	0·36	0·33	0·46	64	19·1	16·7	23	6·3	7·7			
18	12·5	Obstructed	0·05	0·06	1·01	50	18	9·3	2·5	1·0	9·4	120	60	65
		Unobstructed	0·64	0·75	0·7	20·5	11·2	10·3	13	8·3	7·2			

TABLE III.—CHANGES IN CREATININE CLEARANCE DURING PROLONGED ISCHÆMIA OF LIMBS.

Expt. No.	Wgt. of dog, Kg.	Creatinine clearance, c.c./min.			Urine flow, c.c./min.		Blood pressure, mm./Hg.	
		Before.	During.	Per cent. decrease.	Before.	During.	Before.	During.
5	11	29	24	17	0·14	0·17	..	135
6	9·5	29	14	52	0·11	0·06	105	125
9	11	28	5	82	0·08	0·04	125	180
11	11·5	22·5	20·5	9	0·42	0·40	120	150
12	9	14·7	9·2	37	0·40	0·28	115	180
15	23	48	30·5	36	0·13	0·16	110	120
16	15	19·4	10·6	45	0·35	0·59	120	150
17	12	22·5	23	0	0·58	0·36	120	165
18	12·5	30·2	13	58	0·51	0·64	100	120
22	8	22·5	13	42	0·25	0·05	115	185
23	10	34	13·6	60	1·27	0·13	155	165
..	..	32·5	12·3	62	1·14	0·09	..	..

to prevent too drastic a fall in urine flow, which would have rendered collection of samples for analysis difficult.

Chloride and Pigment in Urine after Damage.

Poisoning of tubule cells is usually manifested by inhibition of chloride reabsorption and consequent rise in chloride U/P ratio, in animals such as ours, in which the chloride concentration in the urine is lower than that in the plasma. Table IV shows, however, that there is not that regular rise in U/P ratio as a result of damage which might have been expected. The changes which occurred are probably mainly attributable to the effects of the diuretic agents detailed in the last column of the table.

Urine from the damaged kidney is usually markedly pigmented. We assumed at the time that the pigment was hæmoglobin as unruptured red blood corpuscles could sometimes be seen along with

TABLE IV.—CHANGES IN URINE/PLASMA CHLORIDE CONCENTRATION RATIO DUE TO PROLONGED COMPRESSION AND CRUSHING OF LIMBS (A–B), AND TO THEIR SUBSEQUENT RELEASE (B–C).

A. Before tying limbs.   B. During compression.   C. After release of limbs.

Expt. No.	Wgt. of dog, Kg.	Cl. U/P.			Urine flow, c.c./min.			Change in Cl. U/P.		
		A.	B.	C.	A.	B.	C.	B–A.	C–B.	
2	15	..	0·037	0·16	..	0·126	0·076	..	+0·123	400 c.c. gum saline (C)
4	6·25	..	0·032	0·048	..	0·092	0·15	..	+0·016	320 c.c. gum saline (C)
5	11	0·54	0·12	0·30	0·142	0·173	0·019	−0·42	+0·18	100 c.c. saline (B)
6	9·5	0·7	0·04	0·4	0·108	0·064	0·11	−0·66	+0·36	}440 c.c. gum saline (C)
		0·49	0·042	0·43	0·095	0·089	0·171	−0·45	+0·39	
7	11·5	..	0·14	0·071	..	0·071	0·062	..	−0·07	400 c.c. saline (C)
9 †	11	0·096	0·085	0·23	0·081	0·035	0·18	−0·011	+0·145	2 g. urea (B) 400 c.c. gum saline (C)
10	13	..	0·018	0·023	..	0·25	0·62	..	+0·005	}5 per cent. Na₂SO₄
		..	0·014	0·015	..	0·26	0·61	..	+0·001	} throughout
11	11·5	0·023	<0·008	<0·008	0·42	0·34	0·4	−0·015	0	}5 per cent. Na₂SO₄
		0·02	<0·008	<0·008	0·42	0·35	0·38	−0·012	0	} throughout
12 *	9	0·59	0·007	0·011	0·4	0·28	0·4	−0·58	+0·004	400 c.c. saline 170 c.c. 5 per cent. Na₂SO₄ (B)
15 *	23	0·42	0·17	<0·01	0·13	0·106	0·39	−0·25	−0·16	160 c.c. Na₂SO₄ (C)
16 *	15	0·032	0·017	0·014	0·35	0·59	0·3	−0·015	−0·003	5 per cent. Na₂SO₄ throughout
17 *	12	1·05	0·075	0·013	0·58	0·36	0·33	−0·98	−0·062	500 c.c. saline (A) 300 c.c. 5 per cent. Na₂SO₄ (B)
18 *	12·5	0·55	0·021	<0·02	0·51	0·64	0·75	−0·53	0	500 c.c. saline (A) 250 c.c. 5 per cent. NaHCO₃ (B)
22	8	0·9	0·04	..	0·22	0·04	..	−0·86	..	..

* Ureter pressure raised on other side.   † Limbs ischæmic but not crushed.

casts in the urine. No test was made for the presence of myohæmoglobin. It was noteworthy that the degree of pigmentation of the urine was not parallel with the degree of damage indicated by the reduction in creatinine clearance (Table I).

The Effects of Prolonged low Blood Pressure due to Infusion of Histamine.

It has been suggested by some workers on shock that a variety of organs may be damaged by exposure to low arterial pressure. In three dogs we depressed the arterial pressure to a value of 30–50 mm. Hg by intravenous injection of histamine, and held the pressure at about this value by infusion of histamine at an appropriate rate for $\frac{1}{2}$–1 hour. When the infusion ceased, the arterial pressure at first rose rapidly, but instead of waiting for it to be restored to the pre-histamine value in this way we increased the blood volume by injection of ox serum or gum saline till the pressure had recovered.

During the period of histamine infusion there was no urine flow. After restoration of the arterial pressure, the urine flow recovered to about its original value, and the concentrating power of the kidney was unimpaired as indicated by full recovery of the creatinine clearance. One of the three experiments of this kind is depicted in fig. 3.

Macroscopic Appearance of Kidneys Post-mortem.

The kidneys were notably flaccid, particularly in view of the lavish employment of diuretics toward the end of the experiments. The cut surface showed a yellowish cortex, marked by pale striations at the cortico-medullary junctions, often giving the appearance of a pale zone resembling that described in human kidneys after crush injury. The medulla was usually bluish pink in colour.

Microscopic Appearance of Kidneys Post-mortem.

In a series of 12 dogs with crushed limbs which were bound for a period of 4–5 hours, no specific lesion in any of the various regions of the nephrons could be detected, although there had been an immediate disturbance in renal function demonstrable when the circulation in the damaged limbs had been restored. Since the duration of acute experiments was unlikely to be sufficient for the development of renal lesions comparable with those described in human cases at autopsy, an investigation of the mitochondria in the tubule epithelium was made in all the dog kidneys, with the object of detecting cytological changes which might be correlated with a disturbance in function. A sequence of changes in the shape and staining reactions of mitochondria following cell injury has been described for many tissues in vivo and in vitro, the mitochondria being remarkably sensitive to cell poisons

and trauma. In particular the mitochondria in the epithelium of the proximal convoluted tubules of the kidney have been shown to respond within less than one hour to heavy dosage with poisons such as uranium acetate and sodium oxalate [Gough, 1931]. No significant change in the mitochondria of the proximal tubules of the present series could be found except in one dog which developed irreversible anuria following crushing and ischæmia of its limbs.

The histological appearances of the different regions of the nephron in dogs with crushed limbs were as follows.

Glomeruli and Bowman's Capsule.—The glomeruli in eleven dogs of this series were normal. Foci of atrophic glomeruli or some of the structural changes associated with glomerulo-nephritis were present in the remaining dog. The glomerular capillaries appeared to be intact since there was no evidence of bleeding into the capsular spaces. From the appearance of the glomerular tufts it was impossible to detect any disturbance of capillary circulation. The degree of distension or contraction of the tufts could not be correlated with the flow of urine recorded at the time when the kidneys had been removed. In the one dog which developed irreversible anuria there was more contraction of the tufts than in any other animal of the series..

A non-cellular deposit staining strongly with eosin was found in some of the capsular spaces of all but one dog. This took the form of granular debris or corpuscular masses closely resembling red blood corpuscles. It was impossible to identify the nature of the granular material precipitated by the fixing fluids and the corpuscular masses did not give staining reactions consistent with their being red blood corpuscles.

Proximal Convoluted Tubules.—In all the kidneys examined, this part of the nephron was free from obvious cellular damage. The brush border was intact and clearly defined even in the dog which developed irreversible anuria. Dilatation of the convoluted tubules had occurred in eleven dogs of the series, and this appeared to be independent of the urine flow occurring at the' time of death.. The granular and corpuscular deposit found in the glomerular capsules was concentrated in many of the proximal convolutions, but it was never compacted to form homogeneous casts or what might be regarded as a complete obstruction to the flow of urine, see Pl. I, fig. 8. Ten of the dogs with crushed limbs had been given gum saline or ox serum during the course of the experiments and it seemed not unlikely that some of the debris was from these infusions, see Pl. I, fig. 5. In two dogs with crushed limbs which were not treated with these substances, however, a dense precipitation of similar granular and corpuscular material was observed, and even in apparently normal animals deposits with similar histological appearances are not uncommon.

The mitochondria in the epithelium were in the normal form of

elongated threads packed closely together within the cytoplasm in all dogs except the one which developed irreversible anuria, see Pl. I, fig. 6. In this last dog of the series the mitochondria had broken up into rounded granules, a change indicating an early phase of cell injury, see Pl. I, fig. 7.

The Straight Part of the Proximal Tubules.—The epithelium of this region of the nephron appearing at the junction between cortex and medulla or within medullary rays is formed of large cells which project in a serrated manner on the internal surface of the tubule, so that in the collapsed state the lumen of the tubule is narrow and tortuous. Again in eleven dogs, although the epithelium was normal, much insoluble material had been precipitated within the lumen, see Pl. I, fig. 8. It was this material which often could be seen macroscopically on the cut surface of the kidney to form a series of pale somewhat opaque striations extending from the cortex to the medulla.

The Loops of Henle and the Distal Convoluted Tubules.—From the point where the thin descending limb begins to the end of the distal convoluted tubule, the nephrons were normal and often widely dilated, see Pl. I, figs. 4 and 9. While all the distal convolutions were empty, a few loops, particularly in the papillary zone of the medulla, contained hyaline casts in three of the specimens examined. In contrast with the proximal parts of the nephron, these regions gave the appearance of having been washed through in a remarkably even manner.

The Collecting Ducts.—In three of the dogs a small amount of granular deposit, not sufficient to cause obstruction, was found in some of the papillary ducts. A few ducts in another dog contained desquamated epithelial cells.

The Renal Blood Vessels.—No significant vascular lesions could be found either in the cortex or the medulla. Vasodilation was often marked in the capillaries of the medulla and the veins of the cortex were widely distended in all cases.

Controls.—Three dogs, which had been placed under experimental conditions of anæsthesia and infusion with gum saline or ox serum, were given histamine. Their limbs were not crushed or bound. Sections from the kidneys of these dogs, together with some of unspecified but presumably normal origin, were examined particularly with a view to determining whether the deposits found in the nephrons of the crushed limb series could in any way be regarded as specific for the condition of "crush" anuria. The kidneys of the dogs treated with histamine appeared quite normal except for a heavy deposit of granular and corpuscular material within many of the capsular spaces and throughout almost all the proximal convolutions and the straight proximal tubules, see Pl. I, fig. 5. The distal portions of the nephrons, including the loops, were distended and usually empty. It was quite clear, from an examination of further sections from normal dogs in

which similar insoluble material was again found within the lumen of the proximal regions, that no special significance could be attached to its presence in the crushed limb series, nor could it be regarded as forming in any individual nephron a complete or even substantial obstruction to the flow of urine. It should be emphasised that the degree of precipitation of substances normally present in the lumina of kidney tubules will vary with the composition of the fixing fluid. Aqueous solutions of formaldehyde will reveal rather less insoluble material in sections than the more energetic fixatives such as Bouin's fluid containing alcohol. A somewhat similar variation may be expected in the "structural" appearances of deposits after different techniques of fixation. It would be unwise to assume that any of the coagulable material found in the tubules was protein escaping into the damaged nephrons.

DISCUSSION.

The observations described above have shown that crushing combined with prolonged ischæmia of the hind limbs of an anæsthetized dog injures the kidneys and that the injury persists when the arterial pressure is restored. The injury is characterized by great lowering of the creatinine clearance, the creatinine U/P ratio being low even at low urine flows. The urinary chloride concentration may rise or fall, but does not regularly show the approach to the plasma concentration which is typical of the action of tubule poisons. The urine is scanty and usually pigmented, due to hæmoglobin, myohæmoglobin or their derivatives. The urine contains casts and becomes more acid.

The kidneys are notably flaccid even after brisk diuresis has been induced. The main conclusion drawn from histological observations is that the debris found in the proximal convoluted tubules is not confined to injured dogs or to kidneys which have shown impairment of function. In view of this and its apparent lack of compactness, the debris cannot be regarded as blocking the tubules. This is emphasized by the dilatation and freedom from debris of the loops of Henle, the distal and the collecting tubules and ducts, in kidneys from injured dogs. The damaged kidneys appear fairly vascular and the glomerular tufts are usually rather distended.

The following hypotheses as to the mechanism of the renal damage would account for oliguria and the reduced creatinine excretion when the arterial pressure has been restored.

1. *Blocking of tubules* is not, for reasons just given, suggested as likely by the histological observations. It is incapable of explaining the fall in creatinine U/P ratio at low urine flow, and it would presumably involve a tense kidney, as does a blocked ureter, rather than the flaccid consistency characteristic of kidneys damaged in this series.

2. *Lowered glomerular capillary pressure* would produce effects like

those of adrenaline in doses sufficient to reduce urine flow.   It would account for the flaccidity of the kidneys.   It would account for the increase in creatinine clearance with tubular diuretics, for this phenomenon is observed in uninjured dogs when the arterial pressure is low enough to produce a large reduction in the clearance [Winton, 1934; Shannon and Winton, 1940].   The relatively vascular appearance of the kidneys, and the fact that the glomerular tufts are usually large, are against this hypothesis, and it is substantially excluded by the low creatinine U/P ratio obtaining when the damaged kidneys secrete at a low rate.

3. *Closing down of Glomeruli.*—Variation in the proportion of active glomeruli plays little or no part in variation of renal function in the normal mammal [Winton, 1937; White, 1939], but it might be concerned in the reaction to severe injury.   The observed reduction in creatinine clearance would indicate that two-thirds of the glomeruli were rendered inactive.   The consequences would, presumably, be comparable with those of ligature of one primary branch of the renal artery, and these include a relative increase in urine flow, reduction in the concentration of creatinine and a small increase in that of chloride in the urine [Pickford and Verney, 1929].   These changes are consistent with our observations on the injured dogs.   Against this hypothesis, however, may be cited the vascularity of the kidneys with their large glomerular tufts and the dilated state of the loops and distal tubules.   A serious objection to the hypothesis is the increase in creatinine clearance produced by tubular diuretics, for this phenomenon cannot be demonstrated in normal kidneys except when the creatinine clearance derived from the participating renal units is abnormally low, whereas on this view the clearance from the renal units whose activities survive would be unchanged, or increased.   If the decrease in creatinine U/P ratio is to be explained on these lines, as it is following ligature of a branch of the renal artery, the creatinine clearance in the active renal units would, in fact, be increased owing to the fall in intrarenal pressure [Winton, 1934 a].   At normal clearance, diuretics like urea and presumably sulphate produce, if anything, a reduction in creatinine clearance in the undamaged anæsthetized dog [Eggleton *et al.*, 1940 b].

4. *Increase in intrarenal pressure* which is usually associated with dilated tubules, as observed, invariably involves a tense and not a flaccid kidney, and a reduction in urinary chloride.   This factor can be eliminated.

5. *Poisoning of tubule epithelium* has two main consequences: first, the impairment of reabsorption of water, chloride, etc.; and second, an increase in permeability which allows the constituents of the urine to escape in part from the lumen of the tubule to the venous blood. The isolated perfused kidney poisoned with cyanide or chloral hydrate becomes very tense, and the reduced creatinine clearance is accom-

panied by a spectacular rise in urinary chloride and a polyuria due to inhibition of water reabsorption which is followed, after an hour or two, by oliguria due to increased permeability and intrarenal pressure. It is possible that in the dogs with crushed limbs tenseness of the kidney is not manifested because the organ is innervated. If this be so, this hypothesis would account satisfactorily for the increased creatinine clearance produced by tubular diuretics, and for the changes in the appearance of the mitochondria in the proximal convoluted tubules of the injured dog with irreversible anuria. The lack of a regular and large rise in urinary chloride is, however, a serious objection to this view, and the absence of an initial polyuric phase cannot easily be explained in some of our experiments in which the fall of arterial pressure was small or soon counteracted by replenishment of body fluids.

The hypothesis that an abnormal substance released from the limbs passes into the tubules and is concentrated in them, as is urea, and exerts a poisonous action in virtue of this concentration is rendered unlikely by the fact that the severity of the renal damage is much the same whether anuria be allowed to develop after release of the limbs, or whether this be prevented by the action of diuretics. This hypothesis was rejected as a result of an analysis of the effects of retarding the urine flow by raising the ureter pressure.

6. The possibility that the renal damage is due to exposure to low arterial pressure, and to the consequent *anoxœmia of renal tissue,* is excluded by the experiments on the effects of intravenous infusion of histamine, in which the kidneys recovered fully as soon as the arterial pressure was restored. This possibility is also excluded by observations on three dogs in which after crushing and release of limbs the arterial pressure did not fall below 95 mm. Hg because the blood volume was artificially increased; such a pressure is sufficient for prolonged normal functioning of the kidney, nevertheless, the characteristic renal damage ensued.

It will be seen that no single hypothesis meets the case with entire satisfaction, and it seems probable that more than one of the mechanisms discussed are adversely affected. The renal failure due to crush injuries might well be due, in part, to leakage from the tubules and, in part, to reduced glomerular filtration.

The question as to how closely the lesion described in the kidneys of anæsthetized dogs resembles that in crush injuries in man cannot be finally answered until recovery experiments on dogs allow the later consequences to be studied. It would be fair to say that the effects of crushing limbs in acute experiments are just those which would be expected to be manifest in a period of a few hours if the lesions in the dog and man were the same. The main features of the failure in renal function are clearly the same immediately after the injury in acute

experiments on dogs, and as soon as they have been observed in man. In dogs this type of failure is not associated with some of the histological changes [Shaw Dunn, Gillespie, and Niven, 1941; Bywaters and Dible, 1942] which have been described in human kidneys obtained a week or so after the injury, and on the basis of which theories concerning the primary renal lesion have been developed. Structural changes in the tubular epithelium may be present but not detectable by histological methods in material from acute experiments. Alternatively, the characteristic histological appearances described in the human kidneys may be regarded as secondary changes and not simply related to a primary lesion.

If it be supposed that the renal damage is comparable in our experiments on dogs with that in human casualties, one can say that our observations support the recommendation that diuretics should be given freely because this leads to increased output of urea and other substances. Though diuretics do not by themselves lessen the degree of damage to the kidney, they may prevent death of the patient long enough to enable healing and compensatory processes to become effective. Our experiments were not capable of discriminating clearly between the efficacies of the various diuretic agents employed, and bicarbonate could not be distinguished in its action from that of other diuretics.

SUMMARY.

1. A method of crushing the hind limbs and rendering them ischæmic for 4–5 hours is described which results in impairment of renal function in the anæsthetized dog, and supervenes almost immediately after readmission of circulation to the limbs.

2. The renal damage is characterized by a profound reduction in urine flow and in creatinine clearance maintained even when the arterial pressure is restored by intravenous administration of fluids, by a reduction in urine/plasma creatinine concentration ratio, even when the urine flow is unchanged or reduced, and by pigmentation of the urine. The chloride content of the urine is not systematically affected in these experiments.

3. P.M. the damaged kidney is flaccid. Microscopic appearance of non-cellular debris in the proximal convoluted tubule is shown not to be specifically associated with the deterioration in function of the kidney. The loops of Henle and more distal portions of the nephron are dilated and free of debris.

4. A number of diuretics were shown to be capable of increasing the urine flow from the damaged kidneys, and concurrently to raise the creatinine clearance, but only to about one-half its normal value.

5. The period of anuria ensuing on readmission of the circulation to the limbs is not an essential antecedent of the renal damage. Lower-

ing the urine flow from damaged kidneys by partial obstruction of the ureter does not enhance the damage.

6. The renal damage does not ensue after a considerable period of low arterial pressure maintained by intravenous infusion of histamine into dogs with uninjured limbs.

7. The characteristics of the renal damage, due to limb injury, are discussed in relation to the known renal mechanisms likely to be affected, and it is concluded that the disturbance of no single mechanism could account for all the observed phenomena.

8. During ischæmia of the limbs the arterial pressure slowly rises and the urine flow is apt to fall off. The urinary chloride content falls and the creatinine clearance often also falls somewhat. These changes may be attributed to increase in sympathetic tone.

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SOME PHYSIOLOGICAL APPLICATIONS OF MEASUREMENTS OF FOAM TIME.

By F. Schütz. From the Pharmacological Laboratories, College of the Pharmaceutical Society, University of London, and the Medical School, Hospitals Centre, University of Birmingham.

A NUMBER of physiological phenomena and actions of drugs on cells have been shown to depend on the physical or chemical properties of the substances involved in the process. The surface tension of solutions, for example, has often been found to be correlated with the physiological or pharmacological activity of these liquids. Some of the relationships are known as Richardson's [Richardson, 1869] and Traube's rules [Traube, 1891 and 1904; see also Clark, 1933]. The present paper deals with a newly described physical characteristic of certain liquids and with two of its possible physiological applications.

Measurements have recently been carried out of the stability of foam of various liquids. Foam time (T_f) is an expression of the stability of foam under standard conditions. This was determined for a number of alcohols at different concentrations (c), and T_f and c were plotted against each other. Foam-time/concentration (T_f/c) curves thus obtained offered a number of characteristic features [Schütz, 1942 *a* and *b*], of which only one is dealt with in the present paper.

When foam-time/concentration curves were drawn for a number of aliphatic alcohols in water, it was found that foam time increased with increasing concentration, but only until a certain concentration was reached, when any further addition of alcohol did not further increase the foam time, but decreased it until, eventually, it became zero again at a higher concentration. The T_f/c curves of normal alcohols and of many isomers thus showed a peak (or maximum) of T_f, which, since it proved to be a critical point, was called "critical foam time," and the concentration in the bulk of the liquid at which it occurred was called the "critical T_f-concentration." These critical T_f-concentrations were found to be characteristic for each alcohol and were always found at a lower concentration than 100 per cent.

The critical T_f seems to be the only known physical or chemical constant showing a maximum value at concentrations of alcohols

ing the urine flow from damaged kidneys by partial obstruction of the ureter does not enhance the damage.

6. The renal damage does not ensue after a considerable period of low arterial pressure maintained by intravenous infusion of histamine into dogs with uninjured limbs.

7. The characteristics of the renal damage, due to limb injury, are discussed in relation to the known renal mechanisms likely to be affected, and it is concluded that the disturbance of no single mechanism could account for all the observed phenomena.

8. During ischæmia of the limbs the arterial pressure slowly rises and the urine flow is apt to fall off. The urinary chloride content falls and the creatinine clearance often also falls somewhat. These changes may be attributed to increase in sympathetic tone.

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Considering that the data were obtained from biological experiments, they seem to agree well with the critical T_f concentrations. The surface-tension/concentration curves do not show a maximum at any concentration. Since the critical T_f seems to be the only physical or chemical constant showing a maximum value at concentrations of the alcohols between 0 and 100 per cent., the data shown in Table I suggest that an underlying principle, which is responsible for the greater biological activity of relatively lower concentrations of alcohol, is connected with foam time.

Since some recently described experiments [Schütz, 1942 b] on the critical T_f's showed a certain parallelism to the phenomena occurring during the inversion of emulsions, experiments were carried out to find some other known action of the alcohols which showed a maximum at or near their critical T_f concentrations. In particular, observations were made on emulsification and on the limit interface of globules of lecithin when they are placed in different concentrations of alcohol. Small globules of equal size and weight of technical egg lecithin (B.D.H.) were made and placed in equal quantities of ethyl alcohol of different concentrations. After leaving them standing for eighteen hours at room temperature, one can observe that the swelling effect is greater where less alcohol is present in the solutions, but the higher the alcohol concentration becomes, the more of the lecithin is dissolved (colour of the supernatant liquid). In 100 per cent. alcohol, only a small part of the globule is dissolved, and it is a little diminished in size and not changed in shape. The limit interface, however, between the lecithin globule and the liquid often shows a very distinct picture. This interface is a sharp line at low concentrations as well as at high concentrations of alcohol. In between, however, one can see the formation of a halo around the globules which shows what might be called a spontaneous emulsification. The size of this halo increases from both ends of the series towards the concentration of 55 per cent. At this concentration the halo shows a maximum thickness (1–2 mm.) when the globule has a diameter of about 3 mm. It should be noted that in the solutions containing more water and less alcohol, the limit interface was, notwithstanding the greater swelling of the globule, quite sharp, as it was also in the solutions of higher alcohol content.

When all the tubes in this experiment were shaken, the foam produced around 55 per cent. was extremely stable and did not break for many hours, whilst at 80 or 20 per cent. practically no stable foam could be produced. If a series of such solutions was shaken and the closed tubes left standing for 18 hours in a water-bath at room temperature, the foam layer above the liquid was a few centimetres high in the tubes containing 50 per cent. alcohol; at 40 and 60 per cent. the foam layer was much lower (1–2 cm. ca.), while the tubes with 20, 30, and 70 per cent. alcohol showed only a few bubbles; those

between 0 and 100 per cent. It should be noted that the values of surface tension of alcohol in water fall continuously with increasing concentration of alcohol, and do not show any maximum or minimum between 0 and 100 per cent. The critical T_f appears, therefore, to be a critical point which is not detectable yet by any other physical or chemical means than by measurements of foam time.

1. MAXIMUM FOAM TIME AND MAXIMUM BIOLOGICAL ACTIVITY.

It is known that absolute ethyl alcohol exerts at 100 per cent. concentration much less germicidal power than at lower concentrations [Epstein, 1897; quoted after Topley and Wilson, 1936]. The optimal concentration was stated to be at 50–60 per cent. This fact has been amply studied, confirmed, and also extended for other alcohols (propyl and butyl alcohol) and other organisms by numerous authors [Barsikow, 1901; Hanel, 1900; Hansen, 1907; Igersheimer, 1906; Minervini, 1898; Salzwedel and Elsner, 1900; Weil, 1901; Wirgin, 1903; etc.]. Since this phenomenon is of interest in connection with the problem of the action of drugs on cells, and is not covered by Traube's rule, a number of attempted explanations have been put forward. These explanations (permeability of the cell membrane, diffusion, etc.) appear unsatisfactory, because no physical or chemical characteristic of the alcohols, or mixtures of those with cells or cell materials, could be found which had a maximum or minimum value at a lower concentration than 100 per cent.

This difficulty does not arise in connection with foam time. The critical T_f of ethyl alcohol was found to be at 50 per cent. The strongest germicidal power, as well as toxic potency in certain cases, was found by different authors, working with various germs and animals, to be within the range 45–60 per cent. The critical T_f of propyl alcohol was found to be at 14·6 per cent., while biologically it was found that this alcohol had a much higher potency at a concentration around 30 per cent. than at 100 per cent.

Billard and Dieulafé [1904] found equal maximal toxicities for three alcohols on young fishes at the concentrations stated in Table I, where also the recently found critical T_f concentrations are quoted.

TABLE I.

Alcohol.	Equal toxicity at (per cent.)	Crit. T_f conc. (per cent.).
Ethyl	45	50
Propyl	18	14·66
n-Butyl	9	4·8

dT_f/dc was negative after water had been added to the 100 c.c. mark (i.e. $c >$ critical T_f concentration). Both these conjugate foaming solutions contained, therefore, an equal amount of dye. When vigorously shaken, the foam in A was pink, and whilst the higher layers of it were breaking and so transferring the concentrate to the lower remaining foam layers, the latter became increasingly red. In B, however, the foam was white, or slightly greenish grey. *Even the liquid itself was pink-red in A, and slightly greenish in B.* The difference in colour is best seen with fresh solutions.

It would be, of course, erroneous to assume that in B there was no alcohol in the foam because the latter was not red. This would be neglecting the fact that the dyestuff is also adsorbed, and apparently acting in this case not unlike an adsorption indicator [Fajans, 1935; Kolthoff and Menzel, 1928]. This appears probable, because the colour change is only observed after the solution has been shaken.

It should be noted that the solution which contained *more* of the substance which, in the conception of histologists, is "stained," did not stain when it exceeded the critical T_f concentration.

If a certain tissue does not stain with a certain dyestuff, the substance, in which the dyestuff is known to be soluble, is claimed to be absent from the tissue. The above-mentioned experiment shows that this conclusion may well be erroneous, and suggests that absence of staining may also have its reason in a changed ratio of the substances involved, in which the dyestuff is soluble and insoluble respectively. In the above case, these two substances were alcohol and water.

The described experiment suggests that staining may not only depend on the presence of the substance in which the dyestuff is soluble, but also on the presence of a substance in which it is less or not soluble, and on the ratio between these two substances.

SUMMARY.

The foam stability, when measured under standard conditions, of solutions of alcohol in water was found to increase with increasing concentrations of alcohol, but only up to a certain concentration, when any further addition of alcohol decreased the foam stability. This particular concentration where foam stability was maximal (critical foam-time concentration) was found to be a characteristic for many liquids. The concentrations of alcohols in water which are known to have the highest biological activity (germicidal, toxic) are very similar to these critical foam-time concentrations. Other experiments also suggest that the germicidal or toxic action of alcohols on suspended cells is connected with the same principles which cause the mixtures to produce a more stable foam at the concentrations which are also biologically most potent.

with 10, 80, 90, and 100 per cent. alcohol were completely free from bubbles.

Another observation was made after the shaking. The lecithin solid was emulsified or dissolved readily in the tubes containing the lower concentrations of alcohol, but from 60 per cent. onwards only more vigorous shaking could make all the visible particles disappear (at 60–80 per cent.); but even very vigorous shaking did not emulsify or dissolve the solid completely in the tubes containing 85–100 per cent. of alcohol within a few hours. In these tubes an increasing number of relatively large lumps were seen swimming around and settled down later at the bottom of the tubes.

If a dense emulsion of lecithin in water is added in equal quantities to a series of different concentrations of alcohol, one can see that after gentle shaking the supernatant liquid is far more dense with particles of all sizes at 50–60 per cent. than at lower or higher concentrations. At 30 and 80 per cent. the supernatant liquid is much clearer; at 20 and 90 per cent. it is almost quite clear. Corresponding results on each side of the critical foam-time concentrations were obtained also with propyl and butyl alcohol.

These experiments suggest that the germicidal power of alcohols, or their toxic action on suspended cells, is connected with the same principles which cause the mixtures to produce a more stable foam at the concentrations which are also biologically the most potent.

2. The Significance of Staining with Sudan IV.

In the course of experiments designed to explain the critical T_f [Schütz, 1942 b] it was found that, on both sides of the critical T_f, solutions could be produced which both had the same T_f. A pair of such solutions was called *conjugate foaming solutions*. Two such solutions differ in the concentration of alcohol in water, but have equal T_f's. One such solution is found on any point of the rising part of the T_f/c curve (i.e. where both T_f and c are increasing) and its conjugate on the falling part of the T_f/c curve (i.e. T_f is decreasing, but c is increasing); in other words, dT_f/dc is positive for the former, and negative for the latter conjugate foaming solution.

Sudan IV dissolves in amyl alcohol to give a deep red solution. In water this dyestuff is practically insoluble, and when shaken with water it gives a slight greenish turbidity, the large undissolved particles appearing black. Equal quantities of a saturated and filtered amyl alcoholic solution of the dyestuff were placed in two cylinders (A and B). The amount of alcohol was such as to give a foaming solution where dT_f/dc was positive when filled up with water to 100 c.c. (i.e. c (the concentration of the alcohol) < critical T_f concentration). To cylinder B, pure amyl alcohol was added to the stained quantity of alcohol, so that

THE INFLUENCE OF LYSOLECITHIN AND OF INCUBATION ON THE SHAPE, SIZE, AND FRAGILITY OF ERYTHROCYTES. By W. A. GILLESPIE. From the School of Pathology, Trinity College, Dublin.

THE present paper deals with some experiments concerning the relationship between the morphology of red blood cells and their fragility in hypotonic saline. Following the observations of Haden [1934], Castle and Daland [1937], Dameshek and Schwartz [1940] and others, it is often stated without qualification that the more nearly spherical the red cell, the greater the fragility. From this point of view it was of interest to examine the alterations of the red cells produced on the one hand by the powerful lysin, lysolecithin (prepared by the action of snake venoms on lecithin and probably normally formed in the spleen), and on the other hand by the incubation of unmoving blood samples under conditions resembling those which obtain in the spleen [Bergenhem and Fåhraeus, 1936; Fåhraeus, 1939; Knisely, 1936].

EXPERIMENTAL.

The lysolecithin used in these experiments was prepared by the action of cobra venom on lecithin. After solution of the dry preparation in absolute alcohol it was precipitated by excess ether in the cold, repeated three times. Finally a 2 per cent. stock solution in absolute alcohol was made up. The lysolecithin was freely water-soluble. For use, a certain amount of the stock solution was evaporated to dryness with gentle warmth and the residue dissolved in the required amount of plasma or saline.

The fragility of the red cells was determined by a method essentially the same as that of Creed [1938]. Quantitative fragility curves were obtained, but are omitted from this paper owing to shortage of space.

Lysolecithin and the Fragility of Red Cells.

The first experiments were concerned with the influence of subhæmolytic amounts of lysolecithin on the behaviour of washed red cells in hypotonic saline.

113

An experiment with Sudan IV in alcoholic solutions shows that at the critical foam-time concentration, the staining property of Sudan IV is undergoing a change, and suggests that staining may not only depend on the presence of the substance in which the dyestuff is soluble, but also on the presence of a substance in which it is less soluble, or insoluble, and on the ratio between these two substances.

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taking care to avoid false conclusions from appearances near the coverslip edge.

The results show that the change of red cells to a spherical form induced by lysolecithin is not accompanied by any change in cell volume or fragility. *Stronger concentrations of lysolecithin than were used in this experiment do cause a considerable increase in fragility, but*

TABLE II.—THE INFLUENCE OF LYSOLECITHIN ON THE MORPHOLOGY, VOLUME, AND FRAGILITY OF ERYTHROCYTES IN WHOLE HUMAN BLOOD.

Lysolecithin concentration.	Appearance of erythrocytes.	C. volume (hæmatocrit).	Fragility range (per cent. NaCl).
Nil	Normal; biconcave discs and rouleaux.	30 ml. per cent.	0·42–0·28
1 : 4000	Cells spherical, with prickly surfaces.	30 ,, ,,	0·42–0·28
1 : 3000	Cells spherical, prickles less marked.	30 ,, ,,	0·42–0·28
1 : 2250	Cells spherical, prickles still less marked.	30 ,, ,,	0·42–0·28

this is obviously not due to the spherical form of the cells; it may be due to damage of the cell membrane, perhaps limiting its capacity to be deformed when the cell is placed in hypotonic solutions. These findings correspond with those of Ponder [1937 b] on red cells made spherical by the action of impure lecithin.

Bergenhem and Fåhraeus [1936] showed that the change of red cells to a spherical shape, which occurs in incubated blood, is due in part to a primary change in the cells and in part to a secondary change caused by the lysolecithin which is formed in the plasma. (By applying the term spherocytes to cells made spherical in this way, these authors cause some confusion. This point will be dealt with later.) In order to compare the effects of the primary and secondary changes on the fragility and cell volume, the following experiment was performed.

Fresh oxalated human blood was centrifuged, the plasma removed, and the cells washed twice with saline so as to give finally a concentrated washed cell suspension. Mixtures of 3·5 ml. concentrated cells and 4·0 ml. plasma were made up, the plasma having been treated in various ways as follows:—

A. Cells + normal plasma mixed and kept overnight in refrigerator.

B. Cells + normal plasma mixed and kept overnight at 37° C. without movement.

C. Cells + plasma previously heated to 52° C. for 20 minutes (slight flocculation cleared by centrifuging); mixed and kept overnight at 37° C., without movement.

0·1 ml. of lysolecithin solutions of different strengths in normal saline were added to 0·9 ml. of each NaCl solution in several identical fragility test series. One drop (0·04 ml.) of a washed human red cell suspension (in which the cell concentration was the same as in the original blood) was added to each tube. This experiment was repeated several times with similar results. The results of a typical experiment are shown in Table I.

TABLE I.—THE INFLUENCE OF LYSOLECITHIN ON THE FRAGILITY OF WASHED HUMAN RED CELLS.

Concentration of lysolecithin.	Hæmolysis due to lysolecithin.	Fragility range (per cent NaCl).
Nil (control)	Nil	0·45–0·34
1 : 75,000	Nil	0·41–0·31
1 : 50,000	60 per cent.	

It will be seen that lysolecithin in barely subhæmolytic amounts does not increase the fragility of the red cells in hypotonic saline but if anything diminishes it. The same effect could be shown with sheep and rabbit cells. It was also found that a hypotonic medium diminishes the hæmolytic action of lysolecithin to a certain degree. In producing these results lysolecithin resembles other chemical lysins (sodium taurocholate, phenyl carbamate, brilliant green, etc.) examined by Ponder [1937 a]. Similarly, doses of saponin and hæmolytic serum, large enough to produce some hæmolysis, do not cause increased fragility of red cells [Ham and Castle, 1940].

Morphology, Volume, and Fragility of Red Cells.

The next experiments dealt with the changes in the morphology, volume, and fragility of red cells caused by adding lysolecithin to normal whole blood.

Part of a fresh human blood sample (heparinised) was centrifuged. Different amounts of lysolecithin were dissolved in equal volumes of the plasma and one volume of each lysolecithin solution in plasma was mixed with two volumes of the original whole blood. On the same day the morphology, corpuscular volume, and fragility of the cells in each of the mixtures was determined. A typical experiment is tabulated in Table II.

The corpuscular volumes were determined by Wintrobe's hæmatocrit [1929–30], several hours centrifuging being needed to pack spherical cells to a constant volume. There was no hæmolysis. The cell morphology was determined microscopically between slide and coverslip,

form. That this is not the case (at least when lysolecithin is used) is shown by the following experiment.

Five minutes after the intravenous injection of lysolecithin in saline into a rabbit (0·055 g. per kilo) the red cells were all spherical in shape. At the same time the fragility range had increased from 0·46–0·32 per cent. NaCl to 0·66–0·46 per cent. NaCl. On the following day, however, while the fragility was still raised to nearly the same level, the cells were again biconcave discs arranged in rouleaux. The fragility gradually diminished, but had not returned to its original value after several days, notwithstanding the normal appearance of the cells. Although it may be that on the days following the injection the cell thickness was increased to a degree not obvious on microscopic examination, yet it is evident that the increased fragility could not be attributed to the spherical form of the cells.

The Inhibitory Effect of Plasma on Lysolecithin.

As regards the action of lysolecithin on red cells *in vivo*, it must be remembered that the components of plasma have a marked inhibitory action on the hæmolysis caused by lysolecithin. Thus in a comparative experiment a minimum concentration of 1 : 300 lysolecithin in fresh oxalated blood was required for complete hæmolysis, while a washed cell suspension of the same strength was hæmolysed by a concentration of 1 : 1200. The plasma inhibition depends partly on the alcohol-soluble components (cholesterol, etc.), but mainly on the proteins. An alcoholic extract of serum was six times weaker with regard to inhibition than the original serum. It may be of interest to note that the inhibiting action of normal serum was only slightly diminished by heating to 100° C. (diluted with distilled water and subsequently made isotonic by addition of NaCl), while that of lipoid-free serum [Hewitt, 1927] was much diminished by heating.

The inhibiting action of plasma and the high cell concentration explain the fact that relatively large amounts of lysolecithin must be added to whole blood to render the cells spherical. Still larger amounts are required to cause increase of fragility. When sufficient lysolecithin to produce spherical cells was added to blood it was possible to extract from the plasma an amount of lysolecithin in excess of that which may be found in normal plasma; however, it was never extracted in anything like the full amount added. In these experiments extraction with hot alcohol was apparently superior to cold, while it did not seem to be important to evaporate the extracts *in vacuo* at low temperatures. Evaporation at 50° C. under atmospheric pressure proved satisfactory.

D. Cells kept overnight in refrigerator, plasma at 37° C.; mixed next day.

E. Cells and plasma kept separately in refrigerator overnight; mixed next day.

The effects on morphology, mean cell volume, and fragility are shown in Table III.

TABLE III.—THE INFLUENCE OF INCUBATION OF HUMAN BLOOD ON THE MORPHOLOGY, VOLUME, AND FRAGILITY OF THE RED CELLS.

Blood mixture.	Morphology of red cells.	Mean cell vol. (cu. microns).	Fragility range (per cent. NaCl).
A	Normal; biconcave discs and rouleaux.	72	0·46–0·28
B	Spherical, with rather faint surface prickling.	83	0·54–0·32
C	Nearly all spherical; a few thick discs; prickling as in B.	81	0·52–0·32
D	As in C, but prickling more marked.	72	0·46–0·28
E	As in A.	·73	0·46–0·28

It will be seen that the change of the cells to a spherical form produced by incubation of the blood is accompanied by increased cell volume and increased fragility, whether lysolecithin is formed or not. (Heating plasma as in C prevents lysolecithin formation.) When, however, spherical forms are produced by lysolecithin alone (D), there is no alteration of cell volume or fragility. In further experiments it was found that incubation of washed cells alone also caused a corresponding increase of fragility; control experiments showed that incubation of plasma alone (as in D) is accompanied by the formation of lysolecithin which could be extracted by the method of Bergenhem and Fåhraeus [1936]. The demonstration that increase of fragility and cell volume produced by incubation are dependent on primary cell alterations rather than on lysolecithin formation corresponds with some observations reported recently by Ham and Castle [1940] in a paper which became available only after these experiments were finished.

Effect of Injection of Lysolecithin in the Rabbit.

Bergenhem and Fåhraeus [1936] have recorded that intravenous injection of lysolecithin into a rabbit causes "spherocytosis" and increased fragility. Dameshek, Schwartz, and Gross [1938] obtained similar results in guinea-pigs by injecting lytic sera. In this work the increased fragility of the cells was thought to be related to their spherical

[Lloyd, 1940; Singer, 1941]. The experiments reported in the present paper constitute further evidence that lysolecithin is not the cause of the spherocytosis and increased fragility of congenital hæmolytic anæmia.

Dameshek and Schwartz [1940] have recently concluded that all hæmolytic anæmias are probably due to circulating hæmolysins. While this may be true in some cases, the deductions from their experiments are open to criticism. Having shown that injection of lytic sera into guinea-pigs caused "spherocytosis" and increased red cell fragility, they conclude that the increased fragility results from the "spherocytosis" [Dameshek, Schwartz, and Gross, 1938]. However, these authors make no distinction between the fragile spherocytes of congenital hæmolytic anæmia and the spherical, non-fragile erythrocytes produced by the action of a variety of hæmolysins, including, probably, lytic serum, which as Ham and Castle [1940] point out is without effect on red cell volume or fragility *in vitro*, even in concentrations which cause hæmolysis.

SUMMARY.

1. Lysolecithin added in suitable doses to blood does not increase the red cell fragility in hypotonic saline, although the cells become spherical. The fragility of washed cells is slightly diminished by lysolecithin. The spherical cells produced by lysolecithin are essentially different from the spherocytes of congenital hæmolytic anæmia.

2. After intravenous injection of lysolecithin into a rabbit, the fragility of the red cells remains raised for a much longer period than the spherical shape of the cells can be observed.

3. The effects of incubation of whole blood, and of plasma alone, on the morphology, volume, and fragility of the erythrocytes are contrasted.

4. The importance of the inhibiting action of plasma constituents on lysolecithin hæmolysis is stressed.

5. The bearing of these experiments on some of the problems of hæmolytic anæmia is discussed.

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DISCUSSION.

Castle and Daland [1937] showed that differences between the hypotonic fragilities of the red cells of different mammals can be explained by differences in the dimensions of the cells. The more nearly spherical the cell, the less the volume of water which it can absorb from hypotonic solutions without stretching of its surface membrane and consequent hæmolysis. In several of the hæmolytic anæmias the circulating erythrocytes show an abnormal thickness, thus approaching a spherical form. The term spherocytosis has been applied to this phenomenon, and for the present should be restricted to it. It is probable that the increased hypotonic fragility of the spherocytes of hæmolytic anæmia is at any rate partly the result of their abnormal morphology. [Haden, 1934; Castle and Daland, 1937]. The spherocyte of hæmolytic anæmia is not truly spherical, but is a thickened discoid cell which must be distinguished from the perfectly spherical cells produced by the action of various lysins on normal erythrocytes. Examples of such lysins are impure lecithin and certain dyes [Ponder, 1937 b]; to these may be added lysolecithin. The spherical cells produced by lysolecithin and some other lysins have an unaltered resistance to hypotonic saline, probably because the cell volume is unchanged, so that the amount of swelling which the cell can undergo in hypotonic saline without stretching its surface membrane is undiminished [Ponder, 1937 b].

When blood is incubated at 37° C. without movement, the cells become spherical. In this case the fragility of the cells is increased, probably because of the coincidental increase in cell volume. This increase of volume is caused by the primary alterations of the cells; the secondary changes due to the lysolecithin formed in the plasma do not affect the cell volume or fragility. Ham and Castle [1940] suggest that the primary cell alterations are due to metabolic changes causing an increased concentration of osmotically active substances in the cells. Bergenhem and Fåhraeus [1936] suggested that similar primary and secondary changes may take place in blood stagnating in the splenic sinusoids. Singer [1941] has shown that lysolecithin is formed in the dog's spleen. These phenomena may explain the destruction by the spleen of pathological red cells, and even of normal erythrocytes. The primary cell changes are probably more important than the lysolecithin mechanism. Stephens [1939], however, does not believe that normal red cells are destroyed in significant numbers by the spleen.

The view was adavnced by Bergenhem and Fåhraeus [1936] and Fåhraeus [1939] that congenital hæmolytic anæmia is due to a pathological exaggeration of the action of the spleen on red cells stagnating in the pulp. These authors paid particular attention to the lysolecithin mechanism. Many objections to this theory have been put forward

[Lloyd, 1940; Singer, 1941]. The experiments reported in the present paper constitute further evidence that lysolecithin is not the cause of the spherocytosis and increased fragility of congenital hæmolytic anæmia.

Dameshek and Schwartz [1940] have recently concluded that all hæmolytic anæmias are probably due to circulating hæmolysins. While this may be true in some cases, the deductions from their experiments are open to criticism. Having shown that injection of lytic sera into guinea-pigs caused "spherocytosis" and increased red cell fragility, they conclude that the increased fragility results from the "spherocytosis" [Dameshek, Schwartz, and Gross, 1938]. However, these authors make no distinction between the fragile spherocytes of congenital hæmolytic anæmia and the spherical, non-fragile erythrocytes produced by the action of a variety of hæmolysins, including, probably, lytic serum, which as Ham and Castle [1940] point out is without effect on red cell volume or fragility *in vitro*, even in concentrations which cause hæmolysis.

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ACKNOWLEDGMENTS.

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FURTHER OBSERVATIONS ON THE INTERMEDIATE LOBE PITUITARY HORMONE.

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I. INTRODUCTORY.

EXTRACTS of the pituitary from all classes of vertebrates exhibit melanophore excitant properties. The specific melanophore excitant (B-hormone) is elaborated in the intermediate lobe of species that have a distinct *pars intermedia* and in the anterior lobe of forms without an anatomically distinct intermediate lobe. Work on the rôle of B-hormone in the chromatic physiology of lower vertebrates has been reviewed by Waring [1942].

Landgrebe and Waring [1941] described a new method for preparing a potent "B"-containing extract and compared its properties with those of other "B"-containing extracts.

The first part of the present paper describes (i) the preparation of "B"-containing extracts of still higher purity by a modification of the method used by Landgrebe and Waring [1941], (ii) further observations on the effect of caustic soda on "B"-containing extracts, (iii) results of comparing our products with Stehle's extract. Part II reports further work on the pharmacological effect of "B"-containing extracts and on the fate of the hormone in the animal body.

PART I.

(i) *Preparation of "B"-containing Extracts.*

We have concentrated our attention on two products obtained in attempts to improve the "B"-containing extract previously described. The first is valuable because it is the purest "B" extract so far described and because of its high yield. It also furnished data for a further analysis of the effect of caustic soda on posterior lobe extracts. The second product is useful because the same crude extract can be utilised for preparing extracts rich in oxytocic and pressor properties. Both

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[2] Carnegie Teaching Fellow.
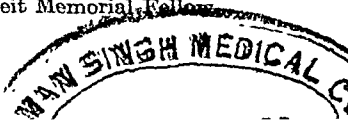
methods are rapid, inexpensive, and have been successfully repeated by independent workers in about 3 hours working to the following instructions:—

Extract (*i*).—(*a*) Mix 5 g. commercial [1] posterior lobe pituitary powder with 50 c.c. of 0·25 per cent. acetic acid in a 150 c.c. Pyrex beaker. Bring the mixture to the boil with stirring over a small Bunsen flame and keep very gently boiling for 5 minutes. Filter hot on a Büchner funnel (Whatman No. 1 paper). Wash powder with further 20 c.c. 0·25 per cent. hot acetic acid and filter. Combine the filtrates.

(*b*) Add 8 c.c. N.NaOH to filtrate thus obtained in a 150 c.c. Pyrex beaker. Bring to the boil with stirring over a small Bunsen flame and keep very gently boiling for 10 minutes. Filter hot on a Büchner funnel (Whatman No. 5 paper). Neutralise filtrate (litmus paper) by adding N.HCl (about 4 c.c.). Cool.

(*c*) Heat to redness in a closed crucible 1 g. powdered animal charcoal, and after 10 minutes heating allow to cool. Add the charcoal to the extract with stirring and leave to stand for 1 hour at room temperature. Filter on a Büchner funnel (Whatman No. 2 paper) till the filtrate is free from charcoal. Wash charcoal with 5 c.c. distilled water and then twice with 10 c.c. acetone. Extract dry charcoal with 20 c.c. glacial acetic acid by shaking in a stoppered bottle for 15 minutes. Filter with suction (No. 2 paper). Add 60 c.c. ether to filtrate and stand for 15 minutes. *Filter (small No. 5 paper). Dry the* precipitate on the paper with a few c.c. of ether. Precipitate weighs 16–18 mg., and 1 mg. contains

<div align="center">

85,000 L.W.[2] units "B."

Less than 1 I.U. Pressor.

  ,,   ,, 1 I.U. Oxytocin.

</div>

On a weight basis this "B"-containing extract is 330 times as active as the original powdered gland from which it was derived.

Extract (*ii*).—If stage (*b*) above is omitted, and acetone is used instead of ether in stage (*c*) as a precipitant, a powder is obtained weighing 20 mg. containing per mg.:

<div align="center">

10,000 L.W. units "B."

Less than 5 I.U. Pressor.

  ,,   ,, 2 I.U. Oxytocin.

</div>

These two extracts are subsequently referred to as L.R.W.$_1$ and L.R.W.$_2$ for the following reasons. There have been many claims for other pharmacological actions of "melanophore expanding hormone" [see Landgrebe and Waring, 1941], but most of these are invalidated because authors have not specified *both* the melanophore expanding potency and the exact method of preparation of their extracts. This should be done for all glandular extracts containing protein. Steroid sex hormones are available as pure chemicals. So when they are injected a simple solution of one chemical substance is involved.

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[1] Prepared by Oxo Ltd.

[2] Landgrebe and Waring [1941]. Pressor and oxytocic activities were assayed on pithed cats and isolated guinea-pig uterus respectively.

Injection of a solution of, *e.g.*, œstrone has more than one effect. If a solution of œstrone is standardised on the basis of its uterus-enlarging capacity, we know the degree of its other effects such as those on the vagina and pituitary. It is quite otherwise with pituitary extracts. No pituitary excitant has ever been obtained in a pure form. We are always dealing with a solution containing various complex polypeptides. So the standardisation of a pituitary extract in terms of one of its activities affords no necessary clue to its other pharmacological properties. For example, some "B"-containing extracts inhibit insulin hypoglycæmia. Other extracts with the same melanophore exciting potency have not this anti-insulin property. Until chemically pure melanophore excitant is isolated we must think in terms of the pharmacological activity of extracts labelled to show *both* the melanophore exciting activity and the method by which the extract was made. The only convenient way to do this is to assign index letters to each extract used and to describe its preparation in detail in the original publication.

(ii) *The Effect of Caustic Soda Treatment on "B"-containing Extracts.*

Caustic soda treatment of unfractionated posterior lobe extracts modifies their melanophore excitant properties in at least two ways: (*a*) the melanophore expanding potency is increased, (*b*) there is an increased duration of response when sub-maximal doses are injected [Landgrebe and Waring, 1941].

The increased potency (*a*) has usually been attributed to destruction of pressor activity by the caustic soda treatment. Landgrebe and Waring (*loc. cit.*) showed that the degree of potentiation brought about by caustic treatment is not correlated with the pressor content of the extract before treatment; and that increased duration of response after caustic treatment may be obtained both from extracts with a high initial pressor content (ox) and extracts with little, if any, pressor properties (dogfish). From these and other data they concluded that "a substance (or substances) other than Jores's 'B' precursor is present in general posterior lobe extracts, which, after treatment with alkali, modifies the melanophore response evoked by 'B' in at least two ways."

There was no evidence at the time to show whether both effects brought about by caustic soda were due to its action on one, or more, substances in the original extract. We now have evidence which (i) suggests that potentiation is a separate process from that causing increased duration of response, (ii) shows that increased duration of response is not an "all or nothing" phenomenon.

The prolonged duration of response after caustic treatment is susceptible of three possible explanations: (*a*) slow absorption of the active principles from the site of injection, (*b*) reduced rate of excretion, (*c*) reduced rate of destruction or of adsorption by the tissues or both.

We have no direct experimental evidence to show which is correct. What evidence there is makes (c) the most probable for the following reasons. *Xenopus* injected with similar doses of caustic soda treated and untreated extracts of comparable purity show only barely significant differences in the speed of initial response [see curves in Landgrebe and Waring, 1941]. So it is unlikely that the difference between prolonged and short responses is due to difference in the speed that the extracts diffuse through membranes (*i.e.* absorption from the site of injection). Experiments described in our previous paper and on p. 138 of this one show that easily measurable quantities of "B" activity are not excreted *via* the kidneys in normal animals. As (c) above is the most likely interpretation of prolonged responses, and until evidence to the contrary is obtained, extracts that evoke prolonged responses may conveniently be referred to as "protected."

New observations on the effect of caustic soda fall under two headings:

(a) *Potentiation of Melanophore Excitant Activity.*—We have confirmed a previous finding that caustic soda treatment of a simple acetic acid extract of our crude ox powder doubles its melanophore expanding power [1] and "protects" it. If carbon is added to such a potentiated and "protected" extract, and elution is effected with glacial acetic acid, a "B"-containing extract is obtained that exhibits only slight "protection" (*i.e.* L.R.W.$_1$). The yield of final product expressed in percentage activity of the original crude extract is 120 per cent. (*i.e.* 60 per cent. yield of the potentiated crude extract). As the amount of melanophore activity in the filtrate after charcoal adsorption represents about 40 per cent. of the activity of the original crude extract, and as a further 30 per cent. can be extracted from the charcoal by further extraction with glacial acetic acid, we can assume that the original crude extract has been fully potentiated by the caustic soda treatment. . Fully potentiated and only partly "protected" "B" may thus be obtained. When phenol is used for elution [Landgrebe and Waring, 1941], potentiated and fully protected "B" is obtained. This suggests that potentiation and "protection" of "B" by caustic soda are separate and distinct phenomena.

L.R.W.$_2$ is a "B" extract prepared by carbon adsorption from crude extracts *not* previously treated with caustic soda. No potentiation is obtained when L.R.W.$_2$ is treated with caustic soda. There is in fact a slight reduction in potency as shown by the following.

Solutions of L.R.W.$_2$ were adjusted to $\frac{N}{10}$ with caustic soda and kept

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[1] We have no knowledge of the lighting conditions to which the oxen were subjected prior to killing. So no data are available relevant to the precursor substance which, according to Jores (1934), accumulates in pituitaries of animals kept in complete darkness and is activated by caustic soda.

in a boiling-water bath for 10 min. and for 60 min.   When cooled and neutralised they were assayed with the following result:—

Before treatment: activity per ml.	After 10 min. treatment: activity per ml.	After 60 min. treatment: activity per ml.
B . 10,000 L.W. units.	B . 8,000 L.W. units.	B . 2,000 L.W. units.
P . . 5 Int. units.	P . less than 0·2 I.U.	P .. less than 0·1 units.
O . . 2 Int. units.	O . less than 0·1 I.U.	O . less than 0·1 units.

These results show a loss of 20 per cent. melanophore excitant activity after 10 minutes treatment and 80 per cent. after 1 hour.

The present state of our knowledge concerning "B" and potentiated "B" (B_1) may be summarised as follows: "B" and B_1 are both adsorbed to charcoal and eluted from it by acetic acid or phenol.   They can be precipitated from these organic solvents by ether or acetone.   Boiling with $\frac{N}{10}$ caustic soda slowly destroys "B."   Similar tests have not been made with B_1.   If the difference between "B" and B_1 is due to the re-arrangements of atoms within the "B" molecule, then adsorption of "B" to charcoal stabilises it so that it can be no longer converted by caustic soda into B_1.   If the conversion of "B" to B_1 involves combination with some other constituent in crude extracts, then the unknown substance is adsorbed to charcoal and is either not eluted by phenol or acetic acid or is not precipitated from these eluents by ether or acetone.   "B" (without B_1) has never been obtained "protected." B_1 can be prepared fully protected (e.g. crude extract treated with caustic soda) or with very little "protection" (L.R.W.$_1$).

(b) *Increased Duration of Response.*—Comparison of duration of the melanophore responses evoked by various caustic treated extracts indicated that there may be different degrees of "protection."   Amounts of different extracts which evoke the same rise of melanophore index show clear differences in the duration of responses obtained (see fig. 1).

When L.R.W.$_2$ is treated with caustic soda there is evidence of very slight "protection" which is even less in degree than that shown by L.R.W.$_1$.   Although for purposes of rough classification L.R.W.$_2$ after caustic soda would be classified as "unprotected," the protection disclosed is slightly greater than could be attributed to destruction of its pressor content alone.

Attempts to isolate the precursor of the protecting agent (X) or the protecting agent itself (X_1) free from "B" have been unsuccessful.

The present state of our knowledge concerning X and X_1 may be summarised as follows.   X is present in crude extracts.   Both X and X_1 when present in original extracts are adsorbed by charcoal.   X is eluted

and precipitated in *very small* quantities by acetic acid and ether (as in preparation of L.R.W.$_2$). Efforts to isolate it from the acetic used for elution, after precipitation of L.R.W.$_2$, have yielded inconsistent results. X_1 is eluted by phenol and can be precipitated with B_1 by acetone. It is partially eluted and precipitated by acetic acid and ether (as in preparation of L.R.W.$_1$).

In our previous paper we left it an open question as to whether oxytocin is X. Tests show that it is not. No evidence has yet been advanced to show that the substance is specifically a pituitary product.
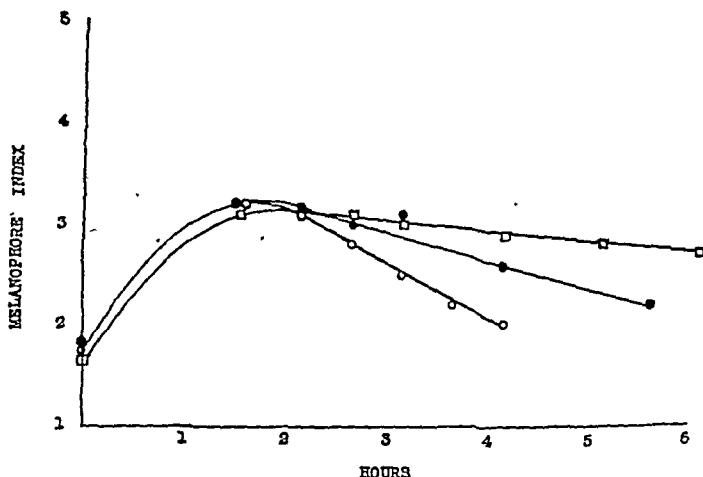

FIG. 1.—Degrees of "Protection" of "B" activity.

Normal *Xenopus*. White background. All injections 1·0 c.c. D.L.S.
-O- = L.R.W.$_1$ (slightly "protected," p. 124).
-●- = Crude caustic treated posterior lobe extract.
-□- = Caustic soda treated "B" powder prepared from *anterior* lobe by original method [Landgrebe and Waring, 1941].

We may merely be dealing with simple inert proteins that delay absorption from the site of injection into circulation [*cf*. Fraser, 1942]. From the time relations exhibited by the response curve it is more likely that "protected" "B" is an extract containing B adsorbed to a *protein* hydrolysate and thus rendered less easily destroyed in the tissues.

(iii) *Comparison of Stehle's* (1936) *Product and our own.*

Broadly speaking, three kinds of activity have been claimed for intermediate lobe extracts: (a) melanophore expansion, (b) erythrophore expansion, (c) "metabolic" effects. We have compared Stehle's product and ours with regard to (a) and (b). Experiments on (c) have been confined to our own extracts. These will be considered separately in the next section.

Professor Stehle kindly supplied us with a sample of his extract of melanophore expanding hormone (1936) prepared as follows:—

A dilute acetic acid extract is concentrated and nearly all the oxytocic and pressor properties are precipitated with ethyl alcohol.   The alcohol solution is evaporated to dryness and the residue extracted with methanol.   Ethyl acetate is added to the methanol solution and the active principle is precipitated. The precipitate is re-extracted with methanol in which the active principle is now insoluble.

(a) *Melanophore Expanding Activity*. — Stehle standardised his powder in frog units.   Our first step was to assay it, using *Xenopus* as the test animal.   We also made rough pressor and oxytocic assays. Table I records the relevant data.

<div align="center">TABLE I.</div>

Preparation.	Melanophore activity.		Pressor activity.	Oxytocic activity.
	L.W. units per mg.	Per cent. of activity in crude extract.		
Stehle . . .	10,000	10	< 1 *	< 1 *
„ after alkali .	20,000	20	0·1	0·1
L.R.W.₂ . .	10,000	16	5	2
„ after alkali .	8,000	13	0·2	0·1
L.R.W.₁ . . .	85,000	120 (see p. 124)	< 1	< 1
„ after alkali	70,000	100	<0·1	<0·1

* Stehle's own assay [1936] was $< \frac{1}{4}$ I.U. pressor and $< \frac{1}{2}$ I.U. oxytocin per 100 melanophore units.   On the assumption that Stehle's melanophore unit is the activity of 0·5 mg. of his standard posterior lobe powder (which on his published data we calculate to contain 200 L.W. units), then 1 Stehle unit = 200 L.W. units. If this is true, Stehle found $< \frac{1}{4}$ I.U. of both pressor and oxytocin in 1 mg. of his powder.

Summarising our results: (i) Stehle's extract is contaminated with the unknown substance X, and is also potentiated by caustic soda treatment; (ii) L.R.W.₂ contains very little X and is not potentiated by caustic soda treatment; (iii) L.R.W.₂ is more highly contaminated with pressor substance than is Stehle's extract.

(b) *Erythrophore Expanding Activity*.—General posterior lobe extracts cause expansion of erythrophores as well as melanophores. Zondek and Krohn were not satisfied with frogs as test animals for "Intermedin."   They introduced the ventral reddening of *Phoxinus* for assay purposes.   A unit of activity was defined as the amount which would cause red patches of 4–9 sq. mm. to appear on *Phoxinus* between the pectoral and pelvic fins and round the anal fins.   Of their most potent preparation 0·001 mg. powder was equal to 1 unit.   The

objections to this test for quantitative work have been fully discussed elsewhere [Landgrebe and Waring, 1941].   The value of the *Phoxinus* response in the present context is that it affords another physiological test by which the Stehle and our intermediate lobe products may be compared.

Apparently Zondek and Krohn did not consider the possibility that the substance responsible for the erythrophore effect in *Phoxinus* might be different from that which evokes melanophore expansion in amphibia.   There is some evidence that two separate excitants are involved.   Some of the evidence is unconvincing [Landgrebe and Waring, 1941], but one observation by Dr. Astwood recorded by Stehle [1938] seemed to offer a good basis for comparing the pharmacological properties of Stehle's preparation and L.R.W.$_1$.

Astwood observed that 0·001 mg. of a preparation similar to Stehle's causes a degree of reddening in *Phoxinus* equivalent to 1 *Phoxinus* unit [Zondek and Krohn].   On a weight basis, therefore, Astwood's preparation and Zondek and Krohn's are equally potent.   Alkali treatment of Astwood's preparation potentiated its melanophore properties, but reduced its erythrophore excitant properties to about one-tenth of the untreated material.

We injected Stehle's preparation (not treated with caustic soda) and L.R.W.$_1$ into *Phoxinus*.   The weight of Stehle powder first injected was the same as that found by Astwood to contain 1 *Phoxinus* unit. The amount of L.R.W.$_1$ injected contained the same number of L.W. melanophore units (Table II).

TABLE II.

Same 12 *Phoxinus* used for all injections.

	L.W. melanophore units.	No. showing ventral red patches.
Stehle Preparation.		
1. ·001 mg. fish	10	8
2. ·005 mg. fish	50	7
L.R.W.$_1$ Preparation.		
1. ·00012 mg. fish	10	8
2. ·0006 mg. fish	50	8

The results confirmed the conclusion previously reached by Landgrebe and Waring [1941] that Zondek and Krohn's method is useless for quantitative assay, and even as a qualitative test it is unreliable.

The 4 animals that did not respond to ·001 mg. of Stehle's preparation were unresponsive to five times the dose. The same 4 animals were refractive to L.R.W.₁. The only definite conclusion that can be drawn from the test is that L.R.W.₁ (subjected to caustic soda treatment during preparation) contains erythrophore activity.

PART II.

(i) "*Metabolic Effects*" of Posterior Lobe Extracts.

A large number of reports have recently appeared describing what may be conveniently referred to as metabolic effects arising after injection of posterior lobe extracts. Some of these have been observed after injection of extracts substantially free from pressor and oxytocic properties, *e.g.* Stehle's melanophore preparation [O'Donovan and Collip, 1938]. In investigating whether any of these effects are evoked by our "B"-containing extracts we have so far confined ourselves to those relating to blood-sugar.

(a) *Previous Work.*

The following effects on blood-sugar have been attributed to various posterior lobe extracts:—

(i) *Hyperglycæmia or Hypoglycæmia in otherwise Untreated Animals.*—The literature to date permits no definite conclusion as to what specific constituent of posterior lobe extracts is responsible for the hyperglycæmic or hypoglycæmic actions of general posterior lobe extracts. The available data imply that: (1) the effects are not attributable to the pressor, oxytocic or melanophore expanding activities as such; (2) they may be associated with any of the above three according to the method of their preparation; (3) the site of injection has important effects; (4) if the effects are due to specific substances as distinct from a mixture of proteins, the fact that the same extract may be effective in one species and not in another, means that to be effective the substance(s) must be injected in a definite combination with protein for each species.

(ii) *Antagonism to Hypoglycæmia evoked by Insulin Injections.*—Experiments on the injection of insulin and posterior lobe extracts indicate that the latter may antagonise the hypoglycæmic action of the former in at least two ways. Burn [1923] showed that when whole posterior lobe extract and insulin were both injected subcutaneously and simultaneously hypoglycæmia does not occur. Gurd [1934] obtained 40 per cent. abolition of insulin hypoglycæmia in rabbits by simultaneous subcutaneous injection of 30 units Pitocin, and 80 per cent. abolition by 30 units Pitressin. If the pituitrin is injected some hours prior to insulin injection it has no inhibitory effect [Young, 1938]. Griffith [1941] as a result of experiments employing posterior lobe extract (Infundin) injected subcutaneously, and insulin injected subcutaneously or intravenously, has concluded that hypoglycæmia does not occur after subcutaneous administration because peripheral vasoconstriction prevents the insulin reaching the circulation in adequate amounts.

As against this type of indirect action some workers have reported that posterior lobe substance can antagonise the effect of insulin *when both are injected direct into circulation.* Here again it is not possible to assign this inhibitory

objections to this test for quantitative work have been fully discussed elsewhere [Landgrebe and Waring, 1941]. The value of the *Phoxinus* response in the present context is that it affords another physiological test by which the Stehle and our intermediate lobe products may be compared.

Apparently Zondek and Krohn did not consider the possibility that the substance responsible for the erythrophore effect in *Phoxinus* might be different from that which evokes melanophore expansion in amphibia. There is some evidence that two separate excitants are involved. Some of the evidence is unconvincing [Landgrebe and Waring, 1941], but one observation by Dr. Astwood recorded by Stehle [1938] seemed to offer a good basis for comparing the pharmacological properties of Stehle's preparation and L.R.W.,.

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We injected Stehle's preparation (not treated with caustic soda) and L.R.W., into *Phoxinus*. The weight of Stehle powder first injected was the same as that found by Astwood to contain 1 *Phoxinus* unit. The amount of L.R.W., injected contained the same number of L.W. melanophore units (Table II).

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The results confirmed the conclusion previously reached by Landgrebe and Waring [1941] that Zondek and Krohn's method is useless for quantitative assay, and even as a qualitative test it is unreliable.

rabbits (1·9–2·0 kg.) were used for the adrenalin-pituitary experiments because they exhibited a more consistent hyperglycæmia to adrenalin. The results obtained were confirmed on Belgian hares. All animals were fed on concentrates, bran, and mixed vegetables. They were kept in separate cages at constant temperature. For insulin experiments the animals were put in the laboratory for at least 20 hours before any experiment and were rested for at least two whole days between experiments. No animal was injected with pituitary extract until at least a week had elapsed since its last injection of extract. Blood was drawn from the ear vein, care being taken not to excite the animals.

(ii) *Estimations.*—Blood-sugar was estimated by the Hagedorn-Jensen method. The error with known solutions was not more than about 5 per cent.

(iii) *Extracts.*—*Insulin*—B.D.H. "AB" Insulin (20 u./c.c.).

Adrenaline—B.D.H. Adrenaline hydrochloride solution (0·1 per cent.).

Melanophore hormone ("B")—see legends, figs. 1–5.

(iv) *Results.*

(1) *Effect of "B"-containing Extracts on Blood-sugar when injected alone.*—Fig. 2 shows the effect of subcutaneous injection of a high dose
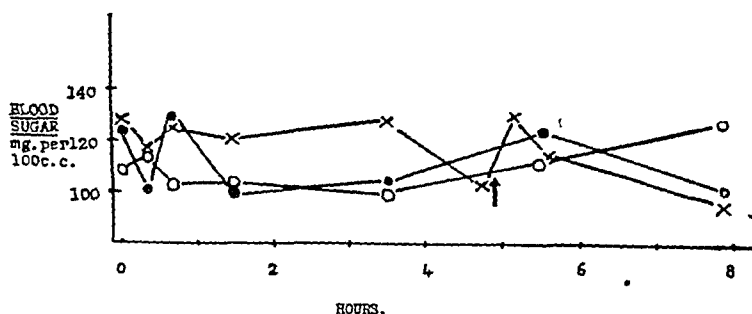

FIG. 2.—Effect of L.R.W.₂ on Blood-sugar.

Rabbits injected (subcutaneously) at 18th hour of fast.

× =Rabbit *A* (male): 50,000 u. "B" at 0 hr.   (3 c.c. extract.)
           50,000 u. "B" at 4¾ hr.   (ARROW.)
-O- =Rabbit *C* (female): 50,000 u. "B" at 0 hr.
-●- =Rabbit *E* (male): 3 c.c. distilled water at 0 hr.   (CONTROL.)

of "B" (L.R.W.₂). No significant change occurred in the treated animals. Slight variations in blood-sugar level occurred in both control and experimental animals.

In the insulin experiments (fig. 4) the initial blood-sugar level of "B"-treated animals was seldom higher than the control figure, except in two cases where the extract was less highly refined. The hyperglycæmia evoked in these two rabbits may have resulted from the

action to pressor, oxytocic, or melanophore excitant activities as such. Thus Geiling, de Lawders, and Rosenfeld [1931] noted "prompt and marked hyperglycæmia" after intravenous injection of Pitocin (1 c.c.) or Pitressin (0·5 c.c.) into insulinised dogs. Ellsworth [1936] found that Post lobin O antagonised insulin hypoglycæmia in the dog, but Post lobin V was ineffective. Pituitary injections were given 5–30 min. after intravenous injection of insulin. Griffith [1941] concluded that "no satisfactory answer can yet be given to the question of the existence of a direct antagonism between insulin and posterior.lobe extract."

Evidence for the possible implication of "B" is derived from two sources. Young [1938] was able to abolish the action of 2 units of insulin (crystalline) given intravenously or subcutaneously to pituitary treated rabbits fasted 21 hours. Anterior lobe extracts were given subcutaneously at the beginning and at the sixteenth hour of the fast. With his best extracts a dose equivalent to about 1–2 mg. dried anterior lobe powder was effective. He was able to show that the "glycotropic" action was not due to prolactin, thyrotropin, or gonadotropin. His extracts were probably contaminated with "B" and he did not eliminate this as the responsible agency. The fact that he found pituitrin ineffective makes it improbable that "B" was the substance involved. Neufeld and Collip [1938] reduced or prevented the hypoglycæmic action of 6 units of insulin in fasted rabbits, by preliminary treatment with an anterior lobe extract or (only one experiment recorded) alkali treated posterior lobe extract. The doses used represented about 0·5–1·0 g. fresh gland tissue. Though they suggested that the anti-insulin effect might be due to "B," no assays for this excitant were disclosed.

(iii) *Antagonism to Hyperglycæmic Action of Adrenaline.*—Relatively little work appears to have been done on the adrenaline "antagonist." Burn [1923] found that subcutaneous injection of posterior lobe extract (Infundin) simultaneously with adrenaline diminished the hyperglycæmic effect of the latter. Gurd [1934] found that adrenaline hyperglycæmia in the rabbit was diminished by doses of 30 units of vasopressin or oxytocin, the former being more effective. Neufeld and Collip [1938] claimed that their insulin-antagonising extracts also antagonised adrenaline in fasted rabbits and concluded that "a more pronounced antagonism to insulin hypoglycæmia and adrenaline hyperglycæmia resulted after prolonged treatment; the adrenaline antagonism appeared to be more defined than that to insulin." In a later paper [1939 b] they reported the effect of posterior lobe extracts in fed rabbits given subcutaneously 45 min. and 0 min. before subcutaneous injection of 0·1 mg. adrenaline. All the extracts—alcohol, ·25 per cent. acetic, and water extracts—were boiled 10 min. with N/10 NaOH to destroy pressor and oxytocin, but apparently no "B" assays were made. Their doses were equivalent to about 1 g. original tissue. Fairly consistent antagonism to adrenaline was observed, even when adrenaline was given intravenously and the extract given subcutaneously or intravenously. Commercial oxytocin was ineffective [Neufeld and Collip, 1939 a].

Young [1938] found that animals rendered insensitive to insulin by anterior lobe extracts were rendered *more* sensitive to subcutaneously injected adrenaline (0·02 mg.) as compared with controls. The action of intravenously infused adrenaline was not significantly altered.

(b) *Experimental.*

(i) *Animals.*—Belgian hares (1·9–2·3 kg.) were used for experiments on blood-sugar effects following pituitary injections alone and for experiments with insulin and pituitary extracts. Havana Rex

received 60,000 units of the "protected" fraction. As with posterior lobe "B," the action of insulin was slightly but not significantly diminished in both animals.
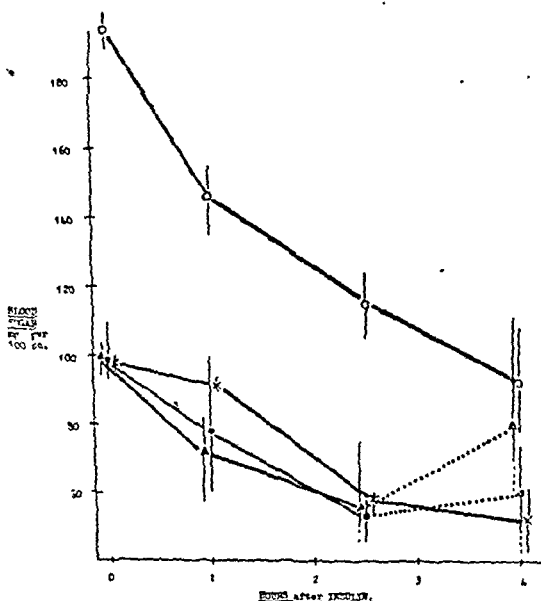

FIG. 4.—Effect of different "B"-containing Extracts on Insulin Hypoglycæmia.

Rabbits (females) fasted 21 hours before subcutaneous injection of 2 units insulin: same pair (C and D) used throughout. "B" injected subcutaneously in two equal portions, 19 and 2¼ hr. before insulin. Vertical lines indicate maximum and minimum values observed.

▲ = 15,000 u. "protected" "B." Two experiments per rabbit—in one case convulsions occurred at 2½ hr.
✳ = 60,000 u. "protected" "B." One experiment per rabbit.
-○- = 75,000 u. "crude protected" "B." One experiment per rabbit.
-●- = Controls (insulin alone). Two experiments per rabbit—each developed convulsions at 2¼ hr. in one experiment.

"Protected" "B"—prepared by eluting from charcoal with N/10 NaOH for 10 min. in boiling-water bath. Pressor content less than 1 I.U./12,500 L.W. units. Contaminated with fair amount of inert protein.

Crude "protected" "B"—original ·25 per cent. acetic extract was salted out by saturation with NaCl precipitate taken up in water, adjusted to N/10 NaOH, kept boiling in water bath for 10 min. and then neutralised. Pressor content less than 10 I.U./75,000 L.W. units.

(c) *Effect of "B"-containing Extracts on Adrenaline Hyperglycæmia.*— Fig. 5 summarises the results of 4 experiments on 3 Havana male rabbits. Experiments were performed at weekly intervals, with the exception that 14 days elapsed between the 2nd and 3rd experiments.

Evidently L.R.W.$_1$ has a definite antagonistic effect on the hyperglycæmic activity of adrenaline. The sequence of experiments precludes the possibility of a developed tolerance to adrenaline. It is noteworthy that the effective weight of extract was similar to the

pressor content (about 10 units) of the extract or from its high protein content. It seems, therefore, that though different "B"-containing extracts may either elevate or depress [*e.g.* O'Donovan and Collip, 1938, using Stehle's preparation] blood-sugar level, this is not a property of all "B"-containing extracts.

(2) *Effect of "B"-containing Extracts on Insulin Hypoglycæmia.*—Since neither Young nor Collip assayed their extracts for "B," any attempt to match the doses of "B" they injected must be very approximate. 1 g. of the sample of "Oxo" anterior lobe·we have in this
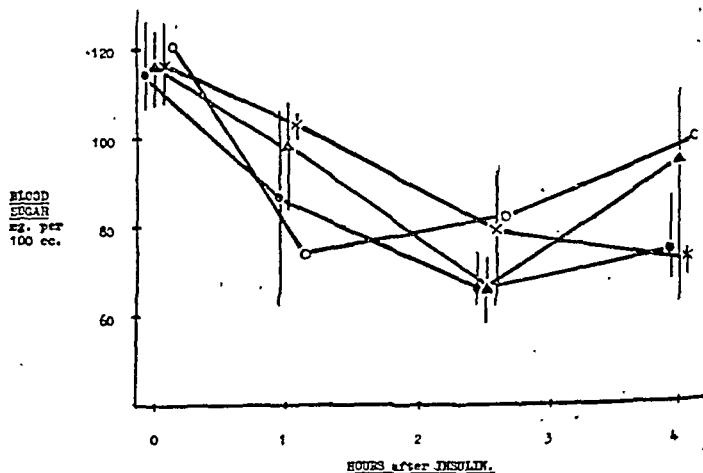

Fig. 3.—Effect of L.R.W.₂ on Insulin Hypoglycæmia.

Rabbits (males) fasted 21 hours before subcutaneous injection of 2 units insulin. Vertical lines indicate maximum and minimum values observed.

▲ =Rabbits *B* (twice), *E*: 35,000 u. "B" INTRAVENOUSLY *immediately before insulin.*
–O– =Rabbit *A*: 35,000 u. "B" SUBCUTANEOUSLY *immediately before insulin.*
× =Rabbits *A*, *E*: 70,000 u. "B" SUBCUTANEOUSLY in two equal doses, 19 *and* 2¼ *hr. before insulin.*
–●– =Rabbits *A*, *B*, *E*: CONTROLS (insulin alone).

laboratory contains approximately 85,000 L.W. units of "B." We used doses of the order of 50,000 units.

Figs. 3 and 4 show that intravenous or subcutaneous injections of highly purified "B" immediately before injection of insulin, or subcutaneous injections 19 and 2¼ hours before insulin, exerted little or no glycotropic effect, though they *slightly* delayed the action of insulin during the first hour. When a crude extract, relatively low in pressor, was injected (fig. 4) the animals exhibited shock symptoms, but there was no conclusive inhibition of the action of insulin.

A "B"-containing extract prepared from anterior lobe powder was used in another experiment. Rabbit *A* received 60,000 units of the unprotected "B," by the procedure detailed under fig. 4. Rabbit *B*

[Heller and Urban, 1935—anti-diuretic from unanæsthetised rabbits; Jones and Schlapp, 1936—pressor activity from pithed cats; Larsen, 1939—oxytocic activity from unanæsthetised rabbits]. All these investigators reported a small percentage recovery in the urine. Heller and Urban [1935] showed that *in vitro* the liver was the most potent tissue for removal of anti-diuretic activity from solutions. Their experiments suggested that the active principle was first adsorbed and later inactivated by enzyme action. Larsen [1939] concluded that aminopolypeptidases from liver and alimentary canal inactivate the oxytocic principle.

We have tested for "B" in the blood, urine, and liver of untreated and injected rabbits.

(a) *Blood.*

1. *Technique for Detecting "B" in Mammalian Blood.*—When injected into the dorsal lymph-sac of *Xenopus*, human or rabbit plasma may cause pronounced local darkening of the skin but only a very slight rise of melanophore index in the webs. This effect is evoked by a number of non-specific agents and so cannot be accepted as evidence for the presence of "B" in the plasma. Quite apart from this, injections of untreated serum are toxic. Death usually occurs ,2 to 3 weeks after an injection. So a means of removing excessive inert protein was devised. Levinson [1940] used a modification of Jores's method. Blood was run into 50 per cent. alcohol, and the filtrate was evaporated to dryness and re-extracted with 70 per cent. alcohol. The filtrate was again evaporated to dryness. After treatment with caustic soda the residue was injected into the test animals. Abramowitz [1937], working with *Fundulus* blood, found that its melanophore expanding potency was increased by caustic soda treatment. The objection to the use of caustic soda treatment of blood is that the resulting degree of potentiation and increased duration of response is not necessarily correlated with the amount of "B" present in different samples.

We first tried the effect of denaturing plasma protein by heat. This did not eliminate the toxic properties sufficiently. We then tried a precipitation technique. The objection to this procedure on general grounds is the inevitable loss of active principle in dealing with small quantities of blood. Since studies of the precipitation of "B" activity from crude aqueous glandular extracts indicated that acetone precipitated most of the active principle we made our first experiments with this agent. Blood was drained into acetone so that the final concentration was about 85 per cent. acetone. The precipitate was dried, ground to a powder, and stored in a desiccator. Extraction with boiling water yielded a toxic solution, and the use of 70 per cent. alcohol was not entirely satisfactory. Extraction with boiling absolute alcohol yielded a solution that contained very little solid matter and

weight of adrenaline antagonised. This contrasts sharply with the amount of pituitary extract used by some workers to inhibit insulin hypoglycæmia. The graph shows that adrenaline evokes a consistent hyperglycæmia in the Havana Rexes. Experiments with Belgian hares indicated that the response to adrenaline was not so consistent and that larger doses of a less refined "B" extract than L.R.W.₁ were
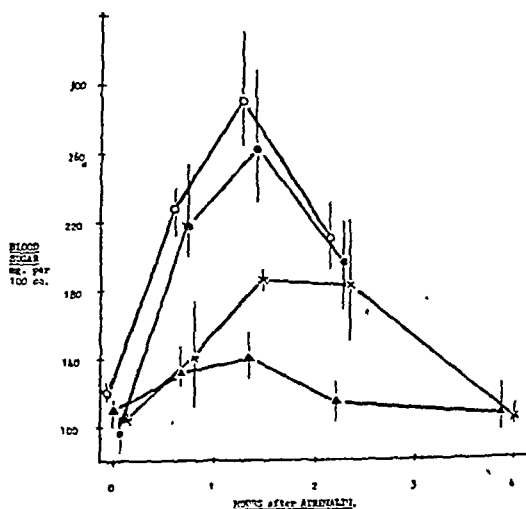

FIG. 5.—Effect of L.R.W.₁ on Adrenaline Hyperglycæmia.

Group of 3 male rabbits, fasted 21 hours before subcutaneous injection of 0·1 mg: adrenaline. "B" injected subcutaneously in two equal portions, 45 *min.* and 0 *min. before adrenaline.*

Vertical lines indicate maximum and minimum values observed.
The experiments were performed in the order listed below.

● =CONTROLS (adrenaline alone).
× =10,000 u. "B" as above.
○ =CONTROLS (adrenaline alone).
▲ =20,000 u. "B" as above.

needed to inhibit the hyperglycæmia. The antagonistic action of L.R.W.₁ is not due to direct chemical inactivation of adrenaline. This is shown by its failure to inhibit a rise of blood-pressure evoked by adrenaline. Experiments described on p. 139 show that an activity indistinguishable from "B" can be obtained from the liver and suggest that the liver can adsorb the activity. So it may be that the anti-adrenaline action of L.R.W.₁ is exerted in the liver (*cf.* Himsworth and Scott's [1938] experiments which show that the action of Young's glycotropic extracts is exerted partly on the peripheral tissues).

(ii) *The Fate of B-hormone in the Animal Body.*

Several investigators have reported the presence of posterior lobe activities in the urine after intravenous injection of pituitary extract

powder imply that he was dealing with small differences based on degrees of melanophore expansion which might be easily produced by substances other than "B."

Levinson's data are of particular interest because he used very small quantities of blood (0·3 c.c.). His technique, however, is open to serious criticism. He treated all his blood extracts with caustic soda and determined the relative potency of various extracts by the *length of time* pale frogs remained dark after injection. The objections to the use of caustic soda in this way have been stated already. There is special objection to its use when the index of activity is the duration of response. Assays based on the *macroscopic* colour of amphibians are quite unreliable. If *Xenopus* or *Rana* are kept on a white background for long periods there is an absolute loss of pigment. Conversely on a black background there is a pigment gain. So frequently a frog with fully expanded melanophores appears macroscopically paler than another with melanophores almost fully contracted. In our experiments a very noticeable darkening frequently occurred and persisted for various times, even though the melanophore index did not rise above 2·5. The reason why we are doubtful as to whether we ever detected " B " in the blood of normal animals is that if the equivalent of 3 c.c. raised the index to 2·5, twice the dose had no greater effect. This does not happen with graded doses of pituitary extract.

" B " can be readily detected in blood from amphibia and fish. In these, blood can be transferred direct without treatment from donor to recipient. We are, therefore, unable to eliminate the possibility that the differences found between warm- and cold-blooded animals may be due to difference of assay technique.

3. *Time taken for Injected " B " to Disappear from Rabbit Blood.*— Doses containing up to 10,000 L.W. units of " B " (L.R.W.₁) were injected subcutaneously and intramuscularly into unanæsthetised rabbits (2 kg. approx.). Three c.c. samples of blood were taken at various times and their " B " content assayed by the method described in the previous section. It is doubtful whether measurable quantities were detected in the circulation. The slight rise of melanophore index observed in the test animals was never greater than would be caused by 2 units per ml. blood. We are not confident that the small excitant activity detected was "B." According to Heller and Urban [1935] the volume of circulating blood of the rabbit may be assumed to be 1/13th of its body weight. So in these experiments only 3 per cent. (at the most) of the injected excitant was detectable at any one time in circulation. We have previously shown that intraperitoneal injection of large quantities of protected and unprotected " B " into mice leads to no detectable quantities of "B" in the urine. Since subcutaneous injections evoke a very definite pharmacological effect (p. 133) either " B " itself is not the adrenaline inhibitor or " B " is removed from the

was not toxic to normal or hypophysectomised *Xenopus*. However, powder equivalent to 3–6 c.c. blood yielded little, if any, "B," so we tested the efficiency of the method by adding a known quantity of "B" extract to blood *in vitro*. Immediately the "B" extract was added, the blood was poured into acetone. The yield was about 60 per cent. *Since loss is bound to occur at at least 3 stages in processing—viz. at precipitation, at solution in alcohol, and at resolution in water after evaporating the alcohol solution to dryness—this low yield is not surprising.* Re-extraction of the residue with ¼ per cent. boiling acetic acid and attempts to recover activity from the acetone yielded little.

Another method was also used with similar results. Blood was run into 4 volumes of absolute ethyl alcohol and heated for a few minutes in a boiling-water bath. The extract was centrifuged while hot, the supernatant fluid poured off and evaporated to dryness in a boiling-water bath. The residue was taken up in a few ml. of distilled water and injected immediately.

2. *"B" Content of Blood of Uninjected Rabbits.*—Special metal boxes were used for experiments on the "B" content of blood from rabbits kept under different conditions of illumination. Those for subjecting animals to complete darkness were enamelled dull black inside and fitted with a false bottom for the escape of urine and fæces. Adequate ventilation was provided by holes fitted with light traps. As an additional precaution all experiments were conducted in a dark room. Boxes for providing white or black backgrounds were similar, but provided with overhead electric illumination. The size and ventilation were such that animals could be maintained in them for lengthy periods.

We kept animals under the different conditions for periods up to 3 days. Blood was run from the ear vein direct into the appropriate amount of acetone. In most experiments we used 3 c.c. of blood, but in some cases up to 6 c.c. was drawn. In no case did we get a melanophore response which could *undoubtedly* be ascribed to "B." This has not been the experience of other workers in this field. Jores [1935] reported an increase of melanophore excitant in the blood of rabbits kept in darkness. Levinson [1940] detected a pronounced diurnal fluctuation in the melanophore excitant content of rat blood. Jores [1935] did not detail his technique, but in a previous paper [1933] he described the precipitation of blood by acetone, extraction with dilute acetic acid, evaporation to dryness, solution of the active principle in hot absolute alcohol, evaporation to dryness, solution of the residue in water, and assay on isolated frog skin. The differences he noted between blood samples were recognisable whether the blood extract was subjected to caustic soda treatment or not. We have previously expressed doubt as to the validity of assays based on isolated skin. The data given by Jores for the potency of his blood extracts in terms of standard

may be associated with or bound to different-sized molecules of protein hydrolysates. This seems to be the only possible interpretation of the otherwise contradictory findings of Zondek and Krohn [1932] and Dreyer and Clark [1923] with regard to the relative diffusion rates of "B," pressor and oxytocin through membranes. So we shall not know whether "B" as ordinarily released from the intact pituitary can pass the kidney until we know in what form "B" is normally secreted or until we can augment endogenous secretion into the circulation.

(c) *Liver.*

We removed liver from untreated rabbits and guinea-pigs, macerated and boiled it in water. The filtered extract was well tolerated by intact and hypophysectomised *Xenopus*. The melanophore index of both was raised to 5 by a sufficiently large injection. Unboiled liver extract had no effect, and on cooling a boiled extract the activity is re-adsorbed to some extent.

The ability to raise the melanophore index of a hypophysectomised amphibian to 5 is generally considered to be a specific property of "B," but we had previously examined a human urine which contained a melanophore excitant that fulfilled this requirement, but did not share with the excitant from pituitary powder the property of adsorbing to charcoal. So we attempted to adsorb "B" from liver with charcoal. Four untreated virgin rabbits were decapitated. The gall-bladder and duct were removed from the liver, and after weighing, the liver was ground with sand. Distilled water was added and the mixture was brought to the boil and kept simmering for 3 minutes. Charcoal was added to the filtered extract. Subsequent treatment was similar to that used for extraction of L.R.W.$_1$ except that no caustic soda was used. Table IV summarises the results.

TABLE IV.

Rabbit.	Sex.	Weight of liver.	L.W. units of "B" recovered.
1	Female	30 g.	4000
2	Male	35 g.	100
3	Male	33 g.	50
4	Female	52 g.	2000

These two criteria, ability to raise the melanophore index of hypophysectomised *Xenopus* to 5 and adsorption and elution from charcoal are as far as we can go at present in identifying the excitant substances from liver and pituitary as the same. A larger series of animals would be needed to confirm that liver from males contains less "B" than those from females.

circulation as rapidly as it enters it.   While we lack certain proof, experiments on liver extraction (p. 139), together with the experiments on blood-sugar (p. 134), suggest that the liver may be involved.

After intravenous injection of "B" extracts into unanæsthetised rabbits the activity could be readily detected in blood samples drawn from the opposite ear within a few minutes.   Table III shows that the excitant was rapidly removed from circulation.

TABLE III.

Male Belgian hare (2 kg.)—unanæsthetised.   10,000 L.W. units injected intravenously.

Time after injection.	Amount detected in blood sample. L.W. units/ml.
5 min.	20 (*i.e.* 30 per cent. still present)
15 min.	10
30 min.	2

The figures have been corrected for the known loss in extraction.

These determinations of the rate of disappearance of the active principle are of the same order as those of Heller and Urban [1935] for anti-diuretic activity in unanæsthetised rabbits and of Jones and Schlapp [1936] for pressor and oxytocic activity in pithed cats.

(b) *Urine.*

Urine was removed from untreated male rabbits and injected direct into *Xenopus*.   It had no melanophore expanding activity. One of the same rabbits was injected intraperitoneally with 50 c.c. warm distilled water and then 50,000 units of L.R.W.$_1$ were injected intravenously.   A further 40 c.c. of water was injected intraperitoneally. Urine pressed from the bladder 2½ hours later contained approximately 5000 units of "B."   The assay was made by injecting urine directly into *Xenopus*.   A check test showed that the excitant was adsorbed to charcoal.

In our previous negative findings with intraperitoneal injections, we used unprotected and fully protected "B."   The intravenous injections described here were made with partially protected "B."   We have not yet made comparable tests with intravenous injection of unprotected and fully protected "B."   Such tests might furnish direct evidence for the mechanism of "protection."   The experiment described here was in the nature of a preliminary experiment to test whether it was possible for "B" to pass through the kidney under certain conditions. The finding has probably no *physiological* significance for another reason besides the obvious one, *i.e.* that the "B" content of the circulation was abnormally augmented.   In different extracts "B" activity

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SUMMARY.

1. Modifications of the Landgrebe-Waring [1941] method for obtaining a high yield of potent melanophore expanding hormone ("B") are described.

2. The best product L.R.W.$_1$ contains per mg.—

   85,000 L.W. units "B" 1 Int. Unit pressor.

3. Properties of our new extracts are compared with those of Stehle's [1936] extract.

4. Extracts may be rich in "B" and yet exert no hyperglycæmic or anti-insulin effects.

5. The hyperglycæmia evoked by 0·1 mg. adrenaline is largely inhibited by subcutaneous injection of 0·25 mg. L.R.W.$_1$. It is not claimed that this inhibition is due to "B" hormone itself.

6. No significant amounts of "B" were detected in the blood or urine of untreated rabbits.

7. Aqueous extracts of normal rabbit liver contain a melanophore excitant that, with the tests at present available, is indistinguishable from pituitary "B"-hormone.

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PHOSPHATE, BASE AND HÆMOLYSIS IN STORED BLOOD.

By MONTAGUE MAIZELS.   From the South-Eastern Blood Supply Depot.

THE present paper falls into two parts: the first deals with chemical and physical changes occurring during storage and the comparison of so-called *in vitro* and *in vivo* tests.   The second part attempts to explain some of the ways by which these changes are brought about.

PART I.

EFFECTS OF STORAGE ON THE COMPOSITION AND SURVIVAL OF ERYTHROCYTES.

Ever since blood banks were first instituted much research has been done to find the ideal diluent which will prevent coagulation of the blood and also give the best possible preservation of erythrocytes during storage. At first, *in vitro* tests were used exclusively, but later methods based on Ashby's [1919] original technique made it possible to study the survival of stored cells in the recipient's circulation [Wiener and Schaefer, 1940; Bushby, Kekwick, Marriott, and Whitby, 1940; Maizels and Paterson, 1940; Mollison and Young, 1940].   As a result of preliminary work, Maizels and Whittaker [1940 *b*] stated that a substance which delayed hæmolysis *in vitro* might not have much effect on survival *in vivo*, and later in a memorandum to the Medical Research Council [1941 *a*] the present writer concluded that there was poor correspondence between the various *in vitro* tests, that these did not always correspond with the actual survival of cells after transfusion, and that of all the tests used in assessing the value of various diluents for stored blood "methods of testing *in vivo* are alone conclusive."   Mollison and Young [1941, 1942] came to similar conclusions in papers presenting extensive data obtained both *in vitro* and *in vivo*.

In vitro tests offer this apparent advantage: that a single specimen of blood may be divided and mixed with a number of different diluents so that any variation in a single sample may be attributed to the diluent alone.   With *in vivo* tests, on the other hand, where survival of cells after transfusion is measured, only one diluent can be used for each transfusion and every transfusion tried requires a different blood and a

143

this time potassium diffuses from the potassium rich cells to the potassium poor plasma, while sodium flows in the reverse direction. This blood is then given to a Group A recipient. Before, and at intervals after the transfusion, samples of blood are withdrawn from the recipient and the A and O cells separated by an agglutination technique. The blood withdrawn is mixed with thirty volumes of high titre Group B serum and allowed to stand for two hours while agglutination occurs. The mixture is then gently agitated and left for a few minutes so that the larger agglutinates may settle. The supernatant fluid is then passed through a filter paper (Whatman's No. 4) to remove the smaller agglutinates and the filtrate containing chiefly free Group O cells is centrifuged and its deposit analysed. Findings in a typical case are shown in Table I.

TABLE I.

Recipient.					Donor cells.	
Cells.		Plasma.				
K m.eq.	Na m.eq.	K m.eq.	Na m.eq.		K m.eq.	Na m.eq.
93	17	3·6	144	Before transfusion .	47	55
				1 hr. after transfusion .	44	60
				24 hrs. ,, ,, .	76	34
				48 ,, ,, ,, .	82	26

It will be seen that K in the transfused cells rises from 47 m.eq. to approach the value in the recipient's cells, although external K is only 3·6 m.eq. while cell Na falls from 55 m.eq. to 26 m.eq., although external Na is no less than 144 m.eq.

With this process of chemical "reconditioning" in mind it is possible to proceed with a consideration of *in vitro* tests.

In Vitro Tests on Blood mixed with various Preservatives.

20 c.c. lots of pooled heparinised blood from two donors were mixed with the following citrate solutions:—

(1) Simple; Na citrate 2·5 per cent., 5 c.c.

(2) Weak glucose: Glucose, 2·8 per cent. in Na citrate 2·5 per cent., 5 c.c. (This solution is similar to the Medical Research Council, 1940, recipe.)

(3) Medium glucose: Glucose 11·2 per cent. in Na citrate 2·5 per cent., 5 c.c.

(4) Rous-Turner: [1916] Glucose: Glucose 3·85 per cent. in Na citrate 1·08 per cent., 46·5 c.c.

new recipient. For these reasons, large series and statistical analyses are necessary if results are to be conclusive.

It is, perhaps, their apparent simplicity and the well-marked differences that they seem to show between cells stored with various diluents that make *in vitro* tests so attractive as a measure of the excellence or otherwise of new preservatives. The information obtained by the use of these tests, however, is so misleading, that a review of the mechanisms on which the tests are based seemed desirable, in order that their limitations might become more clear.

In vitro tests depend primarily on changes occurring in blood cells during storage, delay in such changes being thought to indicate good survival. The changes themselves are of three main kinds: (1) Changes in cell composition due to altering physical equilibria. (2) Degeneration of the cell membrane. (3) Chemical degradation of organic cell constituents.

Changes due to altering Physical Equilibria.

(*a*) Changes in total cell base.

(*b*) Immediate shrinkage or swelling depending on the tonicity of the added diluent, followed by gradual swelling in most cases [Maizels and Whittaker, 1940 *b*; Crosbie and Scarborough, 1941].

(*c*) Spontaneous hæmolysis.

(*d*) Altered fragility [Bagdassarov, 1937; Maizels and Whittaker, 1939; Bushby, Marriott, Kekwick, and Whitby, 1940; etc.].

(*e*) Increase of sodium [Jeannenay, Servantie, and Ringenbach, 1939; Maizels and Whittaker, 1940 *b*].

(*f*) Decrease in cell potassium [Dulière, 1931; Drew, Esdall, and Scudder, 1939; Downman, Oliver, and Young, 1940; etc.].

Degeneration of the Cell Membrane.

(*a*) Spontaneous hæmolysis without cell swelling [Mollison and Young, 1942].

Chemical Degradation of Organic Cell Constituents.

Changes in inorganic and hydrolysable phosphate have been studied.

It is, perhaps, desirable at the outset to point out a serious error in the use of some of these tests: all those in the first group depend on qualitative or quantitative changes in cell base. But such *in vitro* changes are reversible, and in fact chemically abnormal erythrocytes are completely "reconditioned" after transfusion [Maizels and Paterson, 1940]. This "reconditioning" process, which takes place in complete opposition to the concentration gradients, may be demonstrated by the following direct method: Group O blood is stored for a week and during

TABLE II.—CELL VOLUME IN CITRATE SOLUTIONS.

I.			II.		
Na citrate, g. per cent.	Original volume.	Final volume.	Na citrate, g. per cent.	Original volume.	Final volume.
4·2	100	93	4·2	100	93
			3·36	100	96·2
2·8	100	98·5	2·94	100	97·5
			2·52	100	100·4
2·1	100	102·6	2·10	100	101·4
			1·68	100	104·5

The exchange of Na and K across the erythrocyte membrane during storage has already been noted; as a result of citration and the consequent great increase in plasma sodium, Na gained by cells exceeds K lost and cell base (as represented by Na + K) increases. These changes occur in all the solutions tested except Rous-Turner's glucose and Wilbrandt's sucrose. In the former, the immediate effect of citration is to lower plasma sodium from about 145 m.eq. to roughly 120 m.eq., and this results in a gradual decrease of cell base during storage. In the case of Wilbrandt's solution, plasma Na is practically unaltered, but nevertheless a gradual fall of base occurs during storage.

It will have been noted that in the present discussion the effect of glucose or tonicity has not been considered. This is because glucose diffuses into erythrocytes fairly rapidly and the osmotic pressures which it exerts on either side of the cell membrane neutralize one another.

Chemical and Physical Changes in Stored Blood.—The following tests were applied to erythrocytes after storage with the solutions described above: (1) Change of volume expressed as a percentage of the original volume. (2) Potassium content. (3) Sodium content. (4) Total base (Na + K) content. (5) Inorganic Phosphate. (6) Hydrolysable phosphate. (7) Median cell fragility. (8) Spontaneous hæmolysis.

Methods used are described in the appendix.

Figures for the analyses of chemical constituents are referred to unit volume of original cells. Thus if a litre of cells containing 120 m.eq. total base swell to 1·2 litres and have a final content of 110 m.eq., then total base so far from having fallen has actually increased to 132 m.eq. and the actual amount of base in each individual cell has increased by 10 per cent., although the concentration in the cell will have fallen. At present we are concerned with these absolute changes, so that if A is the original volume, B the final volume, and X the content of cells at equilibrium with citrated plasma, then B × X/A is the value used.

(5) Weak acid: [Loutit, Mollison, and Young, 1943] Citric acid
    0·27 per cent. in Na citrate 2·45 per cent. (acid about 0·04 N.),
    5·1 c.c.

(6) Strong acid: Na Citrate 2·5 per cent. in HCl N/10, 5 c.c.

(7) Weak acid glucose: [Loutit, Mollison, and Young, 1943] Citric
    acid 0·27 per cent., Glucose 2·7 per cent. in Na citrate 2·45 per
    cent., 5·1 c.c.

(8) Strong acid glucose: Glucose 2·5 per cent., Na citrate 2·5 per
    cent. in HCl N/10, 5 c.c.

(9) Dextrin: Dextrin 16 per cent. in Na Citrate 2·5 per cent., 5 c.c.

(10) Sucrose: [Wilbrandt, 1940] Sucrose 8·8 per cent. in Na citrate
    1·44 per cent., 21·3 c.c.

Except in the case of the named solutions [Rous-Turner, 1916;
Wilbrandt, 1940] an effort has been made to achieve a solution of
physiological tonicity with a fairly constant sodium content. According
to Crossbie and Scarborough [1942] a solution of 3·1 per cent. Na citrate
has the same osmotic pressure as blood and is, properly speaking,
isotonic. These writers remark that in spite of being isotonic such a
solution may yet produce changes in the volume of erythrocytes, and
they follow Ponder [1934] in distinguishing between isotonic solutions
of the same osmotic pressure and isoplethecontic solutions which though
not necessarily isotonic still cause no change in cell volume when mixed
with blood. These writers state correctly that there is no evidence
that isoplethecontic solutions are superior to isotonic fluids, but in the
absence of proof to the contrary the writer has preferred to use solutions
which cause no apparent change in volume. In some earlier experi-
ments [1939] Whittaker and the writer showed that when erythrocytes
were mixed with one hundred volumes of various citrate solutions no
change in volume occurred in the solution containing 2·1 per cent. Na
citrate. In the present paper, however, it has seemed desirable to
measure changes in cell volume of blood mixed with citrate in the
proportions used in the ordinary citration of blood: 2 c.c. heparinised
blood were centrifuged in each of a number of hæmoglobin comparator
tubes and the volumes of the packed cells noted. To the tubes, 0·5 c.c.
of citrate solutions of various strengths were added and mixed with the
centrifuged blood. After five minutes the tubes were recentrifuged
and the new volumes of cells read (Table II). From this Table it may
be shown graphically that the concentration of citrate may be varied
fairly widely under the conditions of experiment without effecting more
than a small change in the cell volume, although a 2·5 per cent. solution
appears to be that which causes no change in volume.

Such a solution contains 254 m.eq. Na and when mixed with normal
blood in the usual proportions of one part to four will raise the sodium
content of the plasma phase from about 145 m.eq. to about 180 m.eq.

The *in vitro* rankings may be compared with the *in vivo* rankings supplied by Mollison and Young [1942] and Loutit, Mollison, and Young [1943].

TABLE IV.—BLOOD PRESERVATIVES RANKED ACCORDING TO THE RESULTS OF *IN VITRO* TESTS AT 1 AND 4 WEEKS, TOGETHER WITH THE *IN VIVO* RANKINGS OF LOUTIT, MOLLISON, AND YOUNG.

Solutions.	*In vivo* (Mollison and Young).	Percentage volume.		Potassium.		Sodium.		Potassium + sodium.		Inorganic phosphate.		Hydrolysable phosphate.		Median cell fragility.		Spontaneous hæmolysis.	
		1 week	4 weeks	1 week	4 weeks	1 week	4 weeks	1 week	4 weeks	1 week	4 weeks	1 week	4 weeks	1 week	4 weeks	1 week	4 weeks
Rous-Turner glucose	1	9	5	9	8	2	1	9	9	1	1	4	4	7	2	1	1
Weak acid glucose *	1	5	5	2	2	3	4	7	1	5	4	1	1	5	2	1	2
Strong acid glucose	..	8	7	1	1	1	2	6	1	8	4	1	1	9	9	1	2
Weak glucose	3	1	4	4	3	5	5	1	3	1	2	4	4	5	2	1	5
Medium glucose	3	1	3	4	3	5	5	1	3	1	2	4	4	8	8	1	5
Weak acid *	..	5	7	2	3	3	5	1	3	7	4	1	3	3	5	1	7
Dextrin	5	1	2	4	3	5	5	1	7	4	4	7	7	2	6	7	2
Sucrose (Wilbrandt)	6	7	1	8	9	5	2	8	6	9	9	9	9	1	1	9	8
Plain citrate	7	1	7	4	3	5	9	1	7	6	4	8	8	3	6	8	8

* Loutit, Mollison, and Young's solutions [1943].

Results.—The following remarks apply to blood stored at 2°. In general, it will be seen that *in vitro* rankings do not correspond among themselves and that a single test may give different results according to the time which has elapsed before it is carried out. Further, although it might be thought that a solution which helped to maintain normal cell composition during storage would be most likely to ensure survival after transfusion, there is no complete correspondence between the *in vivo* and *in vitro* tests. The original views of the writer [1941 *a*] and of Mollison and Young [1941 : 1942] are thus confirmed.

Percentage Volume.—There is a tendency for erythrocytes to swell during storage. For this reason, hypertonic solutions like Wilbrandt's sucrose which cause initial shrinkage rank low at first and higher later on, while isotonic solutions which cause no initial change of volume lose rank as gradual swelling occurs during storage. Glucose inhibits swelling, while acid solutions cause a greater degree of swelling at first but subsequently delay it—probably owing to their limitation of cation exchange. In Rous-Turner's solution also initial swelling is rapid but the rate slackens later.

Results obtained are shown in Table III which illustrates typical findings after one and two weeks' storage.

TABLE III.—CHEMICAL AND PHYSICAL CHANGES IN STORED ERYTHROCYTES.

Solution.	Days storage.	Volume, per cent.	K m.eq./L.	Na m.eq./L.	Na + K m.eq./L.	Inorganic P, mg. per cent.	Hydrolysable P, mg. per cent.	Median cell fragility.	Spontaneous haemolysis, per cent.
Original blood	0	100	103·0	12·2	115	1·6	11·8	0·38	0
Plain citrate	6	103	69·6	52·8	122	5·8	8·1	0·44	0·3
Weak glucose	6	102	68·2	51·8	120	2·1	8·9	0·44	0·1
Medium glucose	6	99	64·9	48·6	113	1·9	10·5	0·55	0·1
Rous-Turner glucose	6	110	58·2	33·6	92	2·3	9·8	0·55	0
Weak acid	6	106	75·2	40·1	115	6·8	9·3	0·42	0
Weak acid glucose *	6	106	74·0	40·0	114	4·7	9·7	0·43	0
Strong acid	6	108	78·1	34·3	112	23·6	10·3	0·46	0
Strong acid glucose	6	111	86·1	25·2	111	23·0	11·2	0·55	0
Sucrose-Wilbrandt	6	89	47·2	51·2	98	12·6	5·4	0·32	0·6
Dextrin	6	98	63·6	52·0	116	4·6	9·7	0·41	0·1
Plain citrate	12	119	56·0	93·1	149	29·7	4·8	0·53	0·5
Weak glucose	12	107	56·2	69·2	125	5·1	7·3	0·48	0·2
Medium glucose	12	103	56·2	65·0	121	5·1	7·8	0·60	0·2
Rous-Turner glucose	12	109	39·3	43·2	82	5·4	7·1	0·55	0·1
Weak acid	12	111	58·8	60·0	119	26·9	7·6	0·46	0·25
Weak acid glucose *	12	109	61·8	53·8	116	15·4	9·1	0·46	0·15
Strong acid	12	112	69·3	45·1	114	33·2	9·0	..	0·2
Strong acid glucose	12	115	73·6	40·2	114	26·4	8·8	..	0·15
Sucrose-Wilbrandt	12	93	35·3	68·0	103	31·1	5·2	0·36	0·75
Dextrin	12	106	52·1	80·2	132	16·5	4·9	0·46	0·2

* Loutit, Mollison, and Young's solutions [1943].

From the preceding and similar data it is possible to construct a table showing how the various properties of cells deviate from normal during storage and to arrange such deviations in order of magnitude. This has been done in Table IV, where the results of various tests have been ranked so that those which deviate least from the normal are ranked first and those which vary most are ranked last. Thus if the value in fresh cells is 100 and the least variations observed are 90 and 111, then these are ranked first. If the next value is 120 then this will be ranked not second, but third. Unless the differences between the data are definite, they are ranked equal.

In Table IV, then, the rankings of various tests are set out as they are found after roughly one and four weeks' storage. They are derived from eight different experiments and although individual variations occur, the results are on the whole fairly consistent but do not necessarily correspond exactly with the data in Table III.

this dephosphorylation—an action demonstrated by Guest [1932] who summarises the work of earlier workers including that of Rona and Doblin [1911]. In the case of blood stored at 2° this effect of glucose is clearly seen [Maizels, 1941 *b*] and preliminary observations led to the belief that conservation of organic phosphate might parallel survival *in vivo*. It was, however, found that alkalis and fluoride which cause a notable inhibition of dephosphorylation both caused hæmolysis during storage, while acids which accelerate dephosphorylation inhibit hæmolysis *in vitro* [Maizels and Whittaker, 1940 *b*] and also *in vivo* [Loutit, Mollison, and Young, 1943]. Apart, therefore, from shedding some light on cell metabolism during storage, estimation of phosphate has little bearing on problems of cell survival, though it is perhaps worthy of note that after one month's storage very little organic phosphate remains in erythrocytes, whether glucose be present in the diluent or no.

Hydrolysable Phosphate.—About one quarter of the total phosphate in fresh blood cells is easily hydrolysed by a few minutes boiling with normal acid. The residue is fairly resistant to acid hydrolysis. The word "hydrolysable" does not imply that other phosphate is non-hydrolysable, but simply that it is much less easily hydrolysed by acids.

During storage the hydrolysable moiety decreases, and after a month very little may be left at all. Its disappearance is delayed by glucose, and by acids—especially if glucose be present as well. Dextrin and sucrose do not delay the disappearance of hydrolysable phosphate and there seemed some grounds for hoping that the level of hydrolysable phosphate after a fortnight's storage might parallel Mollison and Young's *in vivo* findings. Nevertheless, complete agreement has not been found: alkaline glucose shows better survival of hydrolysable phosphate than neutral glucose citrate solution, although the latter exhibits much less hæmolysis *in vitro*. Again, Rous-Turner's solution preserves hydrolysable phosphate less well than weak acid glucose and no better than ordinary glucose citrate, yet *in vivo* tests [Loutit, Mollison, and Young, 1943] show that Rous-Turner is as good as weak acid glucose and definitely superior to ordinary glucose preservatives.

It is at present true to say that solutions which do not favour cell survival are all associated with the rapid disappearance of hydrolysable phosphate from the cells with which they are mixed. But the reverse is not true, for in the case of alkaline glucose solutions which cause fair survival of hydrolysable phosphate, hæmolysis *in vitro* is rapid. Further, there may well be substances whose preservative effects in other directions far outweigh their inability to help in the maintenance of a high level of hydrolysable phosphate.

Fragility.—In this test, erythrocytes are exposed to a large volume of hypotonic saline. Water can enter the cell so much more rapidly than most osmotically active substances within them can leave, that osmotic

TABLE V.—PERCENTAGE SWELLING OF ERYTHROCYTES IN PRESERVATIVE SOLUTIONS.

Solution.	Percentage swelling at	
	1 week.	4 weeks.
Plain citrate	102–106	125–132
Weak glucose	100–104	118–126
Rous-Turner	104–110	117–125
Weak acid glucose (Loutit, Mollison, and Young) .	104–108	120–125
Wilbrandt's sucrose	88–94	91–96

Potassium.—The K content of cells in fresh blood is high, while in plasma it is low, hence during storage K flows from the former to the latter. Glucose has little or no effect in preventing this loss of K, but acids definitely delay it. The loss may be hastened by increasing the volume of the extra-cellular phase as with Rous-Turner's or Wilbrandt's solutions, when cell K quickly falls to a very low level.

Sodium.—In fresh blood, Na is much lower in cells than in plasma, but during storage an inflow of sodium occurs and cell Na shows a marked increase. This increase is smaller with diluents like Rous-Turner's and Wilbrandt's which are themselves poor in Na. With diluents containing the same Na contents, glucose checks the entry of Na into erythrocytes and acids have an even more marked effect, especially if glucose be present as well.

Sodium + Potassium.—The base of cells from fresh uncitrated blood is in equilibrium with base in the plasma. Citration with most diluents (including that recommended by the Medical Research Council, 1940) increases the base content of the extra-cellular phase from about 150 to 185 m.eq., the change being due to a rise of Na from about 145 to 180 m.eq. For this reason cell base will also usually increase and although cell K falls and Na rises, the rise generally exceeds the fall and the value of Na + K in the cells increases. This increase is very small during the first week of storage, but is definite after two weeks. It is delayed by glucose and especially by acids. Acids, indeed, may cause a slight initial decrease which is, however, not maintained· It is this gradual increase in Na + K occurring with most diluents which explains the swelling of cells during storage and which is partly responsible for their increased fragility. After one month's storage Na + K may increase by more than 20 per cent., and at two months by more than 30 per cent. In the case of Rous-Turner's solution with its low Na content, however, the value of Na + K in cells falls and a similar but slighter fall occurs with Wilbrandt's solution.

Inorganic Phosphate.—During storage, organic phosphate is converted into inorganic phosphate. It is well known that glucose delays

appears to be the more important, for in these fragility is often relatively increased.

In the case of Rous-Turner's solution there is a marked initial increase in fragility, which is doubtless due to the large amount of glucose which the cells take up and which would be more marked were it not for the loss of cell base which this solution induces.   During later weeks of storage, cell base continues to fall and though fragility does not decrease correspondingly, increase, if it occurs, is less rapid than is found with most other solutions.

Wilbrandt's solution resembles Rous-Turner's in that it also causes a decrease in cell base, but it differs in that the sucrose it contains, unlike glucose, diffuses into erythrocytes slowly or not at all.   For this reason the fragility of cells stored with Wilbrandt's solution may be less than that of the fresh original unstored cells—an observation previously made by Mollison and Young [1941; 1942].

From the preceding remarks and from the data in Table IV, it will be evident that fragility has little bearing on the ability of cells to survive after transfusion; and this is to be expected since fragility is merely a measure of osmotically active material within the cells.   Mollison and Young [1942] have proposed to remove one misleading factor from the test by soaking the glucose out of stored cells with normal plasma, before the actual test is done.   They suggest that it may be "possible that changes in osmotic fragility which cannot be reversed *in vitro* are correlated with impaired *in vivo* survival."   It is almost certain that this will not be the case, since the modified test proposed is merely equivalent to measuring the total base in stored cells.   Table IV shows that the maintenance of a relatively unaltered cell base is not correlated with the data for *in vivo* survival which these writers supply.

The fragility test has always been popular with investigators of the properties of stored blood.   Earlier comments made by the writer [1941 a] would still seem to be valid: "This is by far the most misleading of all tests.   The investigator removes the cells from the plasma with which they are in equilibrium and places them in a standard salt solution. If hæmolysis occurs he says that the cells have stood storage badly, and this in spite of the fact that cells with high salt contents are known to be reconditioned after transfusion.   He obtains an absolute measurement of the salt content (or better, osmotically active content) of cells unrelated to the fluid with which they were stored.   This test makes a solution containing glucose-citrate appear little better than simple citrate when in fact it is much superior."   As a corollary of what has already been said, it may be added that the fragility test favours all diluents which have a low total base and glucose content.   For cells stored with such diluents, themselves become poor in osmotically active constituents and correspondingly resistant to hæmolysis by hypotonic salt solutions, even though the diluent itself may cause definite hæmolysis

equilibrium is, in the first case, established almost entirely by the exchange of water. This entails a rapid swelling of the cell, and if the external saline is sufficiently dilute a certain critical value (equivalent to an increase in size of about 50 per cent.) is exceeded and hæmolysis begins. At this point, a further small increase in the dilution of the external salt solution causes a marked increase in hæmolysis. A curve may be plotted of the percentage hæmolysis against the external salt content. The curve is sigmoid and rises sharply from the point where hæmolysis begins to the point where it is nearly complete. From the curve, that strength of salt solution which causes exactly 50 per cent. hæmolysis may be read. This is the median cell fragility of Dacie and Vaughan [1938] and it is a rough epitome of the fragility curve.

If the amount of osmotically active material in erythrocytes is increased, relatively more water is attracted to the cell so that a less dilute salt solution causes hæmolysis and the fragility of the cells appears to be increased. This is what occurs in stored blood where a small increase in total cell base during the first few days of storage is associated with an increase in fragility (Table III), an increase which may be greater than the change in cell base would appear to warrant and which may be due in part to mechanical trauma of stored cells by manipulations incidental to the test. During subsequent weeks, however, the increase in cell base and in fragility is very definite.

In the case of blood stored with solutions containing glucose, the sugar enters the cells and attains equilibrium in a few hours [Klinghoffer, 1940; Maizels, 1941 b]. In this way, osmotically active material within the erythrocytes is increased and, since glucose diffuses outwards more slowly than water can diffuse inwards, it tends to promote hæmolysis when the cells are subsequently exposed to hypotonic salt solutions. For this reason, during the first week of storage, the fragility of cells stored with glucose citrate solution may be greater than that of cells stored with simple citrate. During the second and third week of storage, however, it is probable that the extra amount of sodium taken up by cells stored without glucose, and possibly the excess of inorganic phosphate liberated in them, exert an osmotic pressure far greater than that of the glucose taken up by cells in glucose citrate systems, for after prolonged storage the fragility of cells stored in simple citrate solution is much greater than when glucose citrate is used.

Acid solutions on the whole delay increase in fragility. The buffered cells remain acid even when mixed with a large volume of hypotonic saline and their hæmoglobin will be closer to the isoelectric point. As a result, less cell base will combine with hæmoglobin and more will combine with smaller and more osmotically active anions attracted from the external phase. These changes will tend to increase fragility, but they are counterbalanced by the relatively low total cell base found in weak acid systems. But in strong acid systems the first factor

the same series. If this is done the variance is decreased—except in the case of sucrose systems (Table VII).

TABLE VII.—RATIO OF HÆMOLYSIS OBSERVED WITH VARIOUS DILUENTS TO HÆMOLYSIS OBSERVED IN SIMPLE CITRATE SYSTEMS.

Solution.	2 weeks—11 series.		4 weeks—6 series.	
	Mean.	Variance.	Mean.	Variance.
Plain citrate . .	1	± 0	1	± 0
Weak glucose . .	0·44	± 0·16	0·41	± 0·12
Medium glucose . .	0·44	± 0·17	0·37	± 0·12
Dextrin . . .	0·48	± 0·14	0·36	± 0·15
Sucrose . . .	1·5	± 0·9	0·9	± 0·24

The actual findings differ somewhat from those of Mollison and Young [1942]. According to these authors, sucrose and dextrin have a similar inhibitory effect, both being associated with less hæmolysis than are simple citrate solutions. Tables VI and VII, however, suggest that sucrose is associated with more hæmolysis than simple citrate solution, while the inhibitory effect of dextrin is as great as that of glucose itself. Mollison and Young [1942] also remark that increase of glucose in citrated blood is associated with better preservation *in vitro*, though not with longer survival *in vivo*. In Tables VI and VII, however, the effect on hæmolysis of increasing the glucose content of citrated blood from 0·5 to 2 per cent. is insignificant and, except in the case of dextrin solution, the correspondence between spontaneous hæmolysis and survival *in vivo* is close.

In spite of divergent findings, however, Mollison and Young [1942] confirm the writer's view [1941 a] that spontaneous hæmolysis may give results which do not accord with the findings *in vivo*. Thus in one of my cases, blood stored for three weeks with dextrin solution showed practically no hæmolysis, yet after transfusion, erythrocytes disappeared from the recipient's circulation within forty-eight hours. Apart from this single exception, hæmolysis of bloods mixed with various diluents gives a series which corresponds very well with Mollison and Young's *in vivo* rankings: the superior qualities of Rous-Turner's and of the weak acid glucose solution are exactly paralleled by the slightness of hæmolysis which these solutions cause during storage. But in spite of this, the existence of known exceptions and the possible existence of others unknown, deprive this test of much of its positive value. It has, however, a definite negative value in that an anticoagulant solution causing much hæmolysis is probably valueless. It is suggested as a rough working rule that any new preservative should give less than

in the stored sample. It is therefore thought that the fragility test should no longer be used to measure the value of a diluent as an aid in the preservation of blood.

Spontaneous Hæmolysis.—This test involves the accurate measure of the percentage of cells hæmolysed during storage. Maizels and Whittaker expressed the view in a previous paper [1940 a] that spontaneous hæmolysis was mainly osmotic and that when during storage cells had swelled to a certain critical value (about 50 per cent. greater than their original volume) rupture occurred through a process identical with that found in hæmolysis by hypotonic salines. It was thought that actual degeneration of the erythrocyte membrane might also play a part but that its influence was subsidiary. Mollison and Young [1942], however, from their observations of cell swelling and hæmolysis concluded that osmosis was subsidiary and that cell degeneration was the main factor. Their view is supported by data in Table III, where slight hæmolysis occurs though cells have only swollen by about 10 per cent. and where in some cases after a month's storage hæmolysis was marked although increase in volume was less than 35 per cent.

The data for spontaneous hæmolysis at 2, 4, and 8 weeks is shown in Table VI and also the wide variations due to individual peculiarities

TABLE VI.—PERCENTAGE OF SPONTANEOUS HÆMOLYSIS AFTER STORAGE.

Solution.	2 weeks (11 observations).		4 weeks (8 observations).		8 weeks (2 observations).	
	Mean.	Variance.	Mean.	Variance.	a.	b.
Plain citrate . .	0·7	± 0·39	3·3	± 1·6	4·2	12·2
Weak glucose .	0·27	± 0·14	1·4	± 0·75	3·2	7·0
Medium glucose .	0·28	± 0·20	1·2	± 0·64	2·2	8·9
Dextrin . .	0·31	± 0·18	1·1	± 0·55	0·8	2·1
Sucrose. . .	0·78	± 0·28	2·8	± 1·0	6·0	14·6

in different specimens of blood. Mollison and Young [1941] noted similar wide variations in spite of pooling two or more bloods of identical age.

The findings show the necessity of carrying out large series of observations if different specimens of blood are used and illustrate the advantages of testing out diluents on different samples of the same blood wherever possible. Thus, in Table VI at two weeks there are eleven different bloods, each divided into five samples and mixed with different diluents. The five samples constitute one series, where the percentage of hæmolysis with any particular diluent may be expressed as a ratio of the hæmolysis observed in the simple citrate system of

stability of the cell surface or they might act indirectly by decreasing the activity of lysins.   In connection with the latter possibility it is of interest to consider the effects of pH on lysis due to other agents.

Saponin hæmolysis is increased by acids and decreased by alkalis [Bodansky, 1929], while with complement, lytic action is greatest between pH 7 and 8, decreasing fairly sharply outside these limits [Osborn, 1934].   In the case of soaps, lysolipins, and venoms (which liberate lysolecithin when added to plasma) lysis is inhibited by acids and accelerated by alkalis, the effects within certain small ranges of pH being very marked.   Some of these effects are shown in Table VIII together with the effects of pH on hæmolysis during storage.

TABLE VIII.—pH AND THE ACTION OF VARIOUS HÆMOLYSINS.

Lysin added.	Substrate.	Duration of action.	Temperature.	Per cent. cells hæmolysed at various pH (initial value).			
				6·5.	7.	7·5.	8.
None . . .	Blood (80 per cent.)	14 days	2°	0	0·24	0·36	2·8
None . . .	Blood (7 per cent.)	1 day	16°	0	0·8	2·8	6·0
Cobra venom. .	Blood (7 per cent.)	1 day	16°	8	23	45	73
Na Oleate (0·0025 per cent.)	Erythrocytes (0·5 per cent.)	½ hr.	16°	4	27	85	85
Na palmitate (0·015 per cent.)	Erythrocytes (0·5 per cent.)	½ hr.	16°	10	12	36	90
Saponin (0·00003 per cent.)	Erythrocytes (0·5 per cent.)	½ hr.	16°	27	15	13	11

It will be seen that in the case of cobra venom, which converts the lipins of plasma into lysolipins, the inhibitory effects of a slight fall in pH is qualitatively comparable to the inhibition of storage hæmolysis by acids—a finding which might be expected from the work of Singer [1941] who has shown that after several hours incubation at 37° a substance appears in plasma which may be extracted by methods appropriate to the isolation of lysolecithin and which resembles lysolecithin in its hæmolytic action on erythrocytes.   It might therefore be thought that hæmolysis in blood stored at 2° is due to a more gradual production of lysolecithin at low temperatures.   Lloyd [1941] has shown, however, that hæmolysis may occur in stored blood, even though very little lysolecithin be present.   Two other possibilities remain: hæmolysis in stored blood might be due to an accumulation of soaps, whose lytic action is also inhibited by acids (Table VIII), or it may be due to a direct loss in stability of the elements of the cell surface—a loss

half the amount of hæmolysis observed with plain citrate solution before it is considered worthy of further trial. It is also possible that estimation of hydrolysable phosphate may have a similar negative value and that diluents associated with the rapid disappearance of hydrolysable phosphate are unserviceable. This rule would exclude polysaccharides which delay hæmolysis *in vitro* but do not favour survival after transfusion.

On the whole, however, changes in internal cell chemistry seem to have little bearing on survival during storage and in the circulating blood, and it would seem possible that factors acting on the cell surface may be more important.

pH and Hæmolysis.

Mollison and Young [1942] remark: "Fåhraeus [1939] has claimed that when erythrocytes lose contact with plasma, lysolecithin is formed and the erythrocytes become spherocytic and are then, according to him, half-way to hæmolysis. The development of lysolecithin can be prevented by keeping cells and plasma in contact. . . ." To prevent settling of cells Mollison and Young inverted and mixed blood at intervals during storage but found no improved survival after transfusion. According to Singer [1941], however, lysins developed during storage are probably different from physiological lysins acting *in vivo*, and in this case the mixing technique of Mollison and Young would tend to decrease hæmolysis during storage without necessarily affecting survival *in vivo*. Moreover, it is possible that repeated mixings, however gentle, may damage the erythrocytes and so obscure any beneficial effects exerted by the mixing process. On the other hand, if continued suspension were effected by means other than mixing, then survival might be improved. This may be one of the factors in the superior preservation afforded by Rous-Turner's solution where sedimentation of cells is relatively slow, and where after six days' storage centrifuging still removes a high proportion of residual plasma from the settled cell mass—25 per cent. of the cell sediment, compared with only 13 per cent. in the sediment from ordinary weak glucose citrate systems. Nevertheless, increase in intercellular plasma is not the only factor concerned in preservation by Rous-Turner's solution, for Mollison and Young [1942] have shown that if a large volume of glucose-free diluent is added to blood, preservation is poor, though here also sedimentation is relatively slow.

In the case of acid glucose systems, prevention of "packing" is slight, residual plasma constituting 16 per cent. of the sediment compared with 13 per cent. in non-acid systems. It is therefore necessary to look elsewhere for an explanation of the inhibition by acid diluents of the hæmolysis due to storage [Jeanneney and Servantie, 1939; Maizels and Whittaker, 1940 b]. Thus, acids might act directly by increasing the

stability of the cell surface or they might act indirectly by decreasing the activity of lysins. In connection with the latter possibility it is of interest to consider the effects of pH on lysis due to other agents.

Saponin hæmolysis is increased by acids and decreased by alkalis [Bodansky, 1929], while with complement, lytic action is greatest between pH 7 and 8, decreasing fairly sharply outside these limits [Osborn, 1934]. In the case of soaps, lysolipins, and venoms (which liberate lysolecithin when added to plasma) lysis is inhibited by acids and accelerated by alkalis, the effects within certain small ranges of pH being very marked. Some of these effects are shown in Table VIII together with the effects of pH on hæmolysis during storage.

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Cobra venom. .	Blood (7 per cent.)	1 day	16°	8	23	45	73
Na Oleate (0·0025 per cent.)	Erythrocytes (0·5 per cent.)	½ hr.	16°	4	27	85	85
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Saponin (0·00003 per cent.)	Erythrocytes (0·5 per cent.)	½ hr.	16°	27	15	13	11

It will be seen that in the case of cobra venom, which converts the lipins of plasma into lysolipins, the inhibitory effects of a slight fall in pH is qualitatively comparable to the inhibition of storage hæmolysis by acids—a finding which might be expected from the work of Singer [1941] who has shown that after several hours incubation at 37° a substance appears in plasma which may be extracted by methods appropriate to the isolation of lysolecithin and which resembles lysolecithin in its hæmolytic action on erythrocytes. It might therefore be thought that hæmolysis in blood stored at 2° is due to a more gradual production of lysolecithin at low temperatures. Lloyd [1941] has shown, however, that hæmolysis may occur in stored blood, even though very little lysolecithin be present. Two other possibilities remain: hæmolysis in stored blood might be due to an accumulation of soaps, whose lytic action is also inhibited by acids (Table VIII), or it may be due to a direct loss in stability of the elements of the cell surface—a loss

which might be delayed by a slight fall in pH. In the latter case the similarity of pH effects on hæmolysis due to storage and on lysis by derivatives of the higher fatty acids would be fortuitous.

Inhibition of lysis by acids would also help to explain the preserving action of glucose which produces an acid reaction in stored blood (*cf.* Tables XIII and XIV). But here the explanation is quite incomplete, for simple acidification in the absence of glucose does not preserve blood as well as if glucose be present. It may be remarked that glucose has no inhibitory action on such lysins as lysolecithin and soaps, and it would seem probable that the action of glucose in preventing hæmolysis is not due to its effects on pH alone but to some additional factor, possibly of a nutritional nature.

PART II.

Chemical and Physical Changes in Stored Blood at 2°.

The changes about to be described always occur in one form or another whenever blood is stored, and for this reason the simplest system investigated, that of blood kept with plain citrate solution, will be considered more fully.

Simple Citrate Solution.—Changes of blood stored with simple citrate solution are produced by four main factors: (1) Loss of apparent impermeability of cation. (2) The addition of a relatively large amount of non-penetrating anion—citrate—to the medium external to the cell. (3) Alterations in cell metabolism. Of these, changes in the phosphate cycle have been selected for study. (4) Chemical disintegration or loss of physical stability of the cell membrane.

The distribution of ions in circulating and shed blood is shown in Table VIII, where ions present in small amounts are placed in round brackets.

It will be noted that in the circulating blood, sodium and potassium are described as non-penetrating ions, although it has already been shown that in life cations exchange readily across the cell membrane. However, cations in cell and plasma are set at a fairly constant level which the base exchanges merely serve to maintain. The same is true of organic phosphate in the erythrocytes: this increases in alkalosis, decreases in acidosis [Haldane, Wigglesworth, and Woodrow, 1924; Rapoport, 1937], but remains fairly constant under normal conditions. It must be presumed that these substances along with hæmoglobin and plasma protein act in the intact circulation as non-penetrating ions in relation to which the penetrating ions are distributed.

Since, then, erythrocytes while containing roughly the same amount of non-penetrating cation as plasma, have a large excess of non-penetrating anion (Hb^-, phosphate$^-$, etc.), the distribution of penetrating ions should accord with the Donnan equation. Owing to the complexity

TABLE IX.—PARTITION OF IONS IN BLOOD.

	Cells.	Plasma.
Partition of Ions in Circulating Blood.		
Anion penetration—		
rapid . .	Cl⁻, HCO₃⁻, OH⁻	Cl⁻, HCO₃⁻, OH⁻
absent . .	Hb⁻, Organic phosphate⁻	Protein⁻, (Inorganic phosphate⁻)
Cation penetration—		
rapid . .	H⁺	H⁺
absent . .	(Na⁺), K⁺	Na⁺, (K⁺)
Partition of Ions in Shed Blood at 2°.		
Anion penetration—		
rapid . .	Cl⁻, HCO₃⁻, OH⁻	Cl⁻, HCO₃⁻, OH⁻
slow . .	..	(Inorganic phosphate⁻)
very slow . .	..	Citrate⁻
extremely slow .	Organic and inorganic phosphate⁻	..
absent . .	Hb⁻	Protein⁻
Cation penetration—		
rapid . .	H⁺	H⁺
slow . .	Na⁺, K⁺	Na⁺, K⁺

of the system, however, quantitative agreement is not close [Van Slyke, Wu, and McLean, 1923], but qualitative agreement exists, for in freshly drawn blood erythrocytes have in fact a lower concentration of Cl^- and HCO_3^- than the plasma and are also more acid.

Partition of Ions in Shed Blood.—In the scheme presented above, ions have been graded according to their rates of penetration. The speed with which Cl^- and HCO_3^- penetrate is evidenced by the findings of the ordinary respiratory cycle [Henderson, 1928]. The grading of the other speeds of penetration may be derived from Table X. The differences between columns 2 and 3 give the concentration gradients, while columns 4, 5, and 6 record the absolute gains or losses of the substances considered after 1, 2, and 4 weeks' storage.

In shed blood, then, ions acquire new and different rates of penetration and their consequent distribution is complex and unstable. It is probably simplest to regard the more penetrating ions as penetrating and all relatively slowly moving ions as non-penetrating. Changes during storage may then be divided into four stages: Stage (1), redistribution of H^+, OH^-, Cl^-, HCO_3^- and water, while K^+, Na^+, Hb^-, plasma protein⁻, organic and inorganic phosphate and citrate⁻ all act as non-penetrating ions. Stage (2), Na^+ and K^+ which penetrate slowly, redistribute in relation to ions which compared to them penetrate

TABLE X.—PLASMA-CELL CONCENTRATION GRADIENTS AND ABSOLUTE CHANGES IN THE CONTENTS OF CELL CONSTITUENTS DURING STORAGE AT 2°.

	Initial concentrations per litre of water in		Absolute changes in contents per litre of original cell at		
	Citrated plasma.	Cells.	1 week.	2 weeks.	4 weeks.
Na m.eq. . .	188	17	+40	+81	+104
K m.eq. . .	3	146	-33	-47	- 55
Citrate m.eq. . .	88	0	..	+ 5·5	+ 12·5
Total phosphate mM	1	25	0	- 0·5	- 1

slowly or not at all—citrate⁻, phosphate⁻, Hb⁻ and plasma protein⁻. These changes in cation distribution are accompanied by a secondary redistribution of H^+, OH^-, Cl^-, HCO_3^- and water. Stage (3), a very slow redistribution of citrate occurs governed by Hb⁻, plasma protein⁻, and the almost immobile organic and inorganic phosphate⁻, and accompanied by a redistribution of ions more mobile than citrate⁻ itself. Stage (4), an extremely slow redistribution of organic and inorganic phosphate⁻, conditioned by the completely non-penetrating hæmoglobin⁻ and plasma protein⁻. These changes are, of course, continuous and overlapping: stage 2 overlaps stage 3, while before stage 3 is far advanced and while stage 4 has hardly begun, hæmolysis cuts short further observations.

Stage 1.—This is a very short stage in which H^+, OH^-, Cl^-, and HCO_3^- are regarded as penetrating ions, while all other ions may be considered as non-penetrating. It has already been seen that erythrocytes in freshly drawn blood contain a large excess of non-penetrating anion— hæmoglobin⁻ and phosphate⁻. On citration a large amount of non-penetrating anion, citrate⁻, and an equivalent amount of non-penetrating cation, sodium⁺, are added to the plasma so that the original relative excess of non-penetrating anion in the erythrocytes remains undisturbed.

But citration introduces other factors: Na citrate is alkaline and will tend to raise the pH of blood with which it is mixed. This will cause water and Cl^- to flow from cells to plasma, but as citrate is almost unbuffered at blood pH, changes of reaction in cells and plasma and chloride and water shifts will be extremely small. Then, too, since chloride ions in cells and plasma were originally in equilibrium, dilution of chloride in the external phase by water added along with the citrate will cause a passage of chloride from cells to plasma, and since the chloride ion cannot move unaccompanied and since K and Na cannot penetrate the cell membrane, Cl^- will move in company with H^+. In this way HCl will leave the cells which will become relatively more

alkaline, while the plasma becomes relatively more acid.   The process involves an hydrolysis of neutral salts and only proceeds to a very limited extent, so that passage of water and chloride from cells to plasma due to both of the two preceding causes will be very small.

Actually citration causes a loss of about 2 m.eq. chloride from erythrocytes and about 1 m.eq. of this is due to dilution of chloride in the intercellular plasma by citration, while part of the fall may be due to the change in pH and to dilution of the external phase.

Stage 2.—H^+, K^+, Na^+, OH^-, Cl^-, and HCO_3^- considered as penetrating ions; citrate⁻, phosphate⁻, Hb⁻, and plasma protein⁻ considered as non-penetrating.   In this stage the content of cell K falls owing to the low external concentration of potassium, cell Na rises because of the high external concentration of sodium, while the content of Na + K increases.   At the same time water enters the cell which swells and as a result of the combined changes the concentration of Na + K in the cells during storage is but little altered.

In these changes the chief factors are increase of sodium and the non-penetrating anion citrate in the plasma phase.   It has already been noted that citration increases the sodium content of plasma from about 145 to 180 m.eq., so that total base as represented by Na + K rises from about 150 to 185 m.eq. and the base concentration in a plasma containing 93 per cent. of water rises from 160 m.eq. to 195 m.eq. in a citrated plasma containing 95 per cent. water.   It might be thought that this increase of external base concentration would be accompanied by an increase in the concentration of cell base.   But although the *content* of cell base increases, the concentration does not rise.   The reason for this will be discussed later.

The effect of citration in raising the content of non-penetrating anion in the plasma is shown in Table XI, where it is assumed that non-penetrating anions in cells (hæmoglobin⁻, phosphate⁻, etc.) and in the plasma (protein⁻, etc.) equal $K^+ + Na^+ - Cl^- - HCO_3^-$.   All values are experimental except that for cell HCO_3, which is calculated from the equation $[HCO_3]$ cells $= [HCO_3]$ plasma $\times [Cl]$ cells$/0.8 \times [Cl]$ plasma [Van Slyke, Wu, and McLean, 1923].

The values for non-penetrating anions in the previous table are based on several assumptions and are approximate, but the magnitude of the changes make it clear that the normal relative lack of non-penetrating anion in the plasma of uncitrated blood is replaced by a relative excess after citration.   This change-over must, in accordance with the Donnan equation produce the following alteration: the concentration of base (greater in the cells of uncitrated blood) will become greater in the plasma of citrated blood.   At the same time, as equilibrium is approached the cells tend to become less acid than the plasma, instead of being more acid as in fresh uncitrated blood.   Finally, the concentration of penetrating anion, Cl^- and HCO_3^- originally greater

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TABLE XIII.—CONCENTRATION OF K AND Na IN CELLS AND PLASMA OF BLOOD STORED WITH PLAIN CITRATE SOLUTION AT 2° (pH READINGS AT 17°).

	Cells.				Plasma.			
	pH.	K m.eq.	Na m.eq.	Na + K m.eq.	pH.	K m.eq.	Na m.eq.	Na + K m.eq.
Fresh uncitrated blood	..	146	17	163	..	4·5	154	158·5
Fresh citrated blood	7·54	146	17	163	7·57	3	189	192
Citrated blood at 1 week	7·36	94	71	165	7·37	25	166	191
„ „ „ 2 weeks	..	62	104	166	..	38	156	194
„ „ „ 4 „	7·15	46	114	160	7·09	49	152	201
„ „ „ 8 „	..	42	124	166	..	53	146	199

Table XIII shows that cells originally more acid than plasma gradually become relatively less acid—although the acidity of both phases actually increases. It also shows that citration increases plasma base to a concentration which is maintained at a fairly constant level during two months' storage.

During the first two weeks of storage, the concentration of cell K gradually falls but still remains greater than the concentration of plasma K. The figures suggest that sometime in the second month of storage cell K falls below plasma K and that cation equilibrium is approached, when according to the Donnan equation

$$[K] \text{ cell}/[K] \text{ plasma} = [Na] \text{ cell}/[Na] \text{ plasma} = [K + Na] \text{ cell}/[K + Na] \text{ plasma}.$$

The observed ratios are 0·8, 0·85, and 0·83. The agreement is sufficiently close in view of all the experimental errors and of the possibility that the activities of Na and K are not necessarily the same in either cell or plasma.

Since during storage, the concentrations of base alters but little, it may seem paradoxical that both water and base should continue to enter the cells in increasing quantities. But this process is a direct result of citration which suddenly raises Na and hence total base in the plasma and at the same time adds a large amount of non-penetrating anion—citrate—to the extra-cellular phase. The resulting Donnan equilibrium requires that an excess of base remain in the citrated plasma, while an excess of penetrating anion be turned over to the cells, and that all this should take place without disturbing the osmotic equilibrium. The requirements can only be satisfied by the passage of water and anion, and of base in relatively low concentration from plasma to cell. Theoretically, it should be possible to calculate the equilibrium values for cell and plasma base, acid and water from the analyses of fresh blood, but this would involve the following approximations: (1) Osmotic pressure of hæmoglobin. (2) Osmotic pressure of cell phosphates. (3) Amounts, distribution, and osmotic pressure of

in the plasma than in the erythrocytes will ultimately become greater
in the cells than in the plasma.

Some of these changes are illustrated in Table XIII which, apart
from pH data, is derived from the figures in Table XII. The pH data,
which are discussed later in the section on methods, have a relative
rather than an absolute meaning.

TABLE XI.—CONCENTRATION OF IONS IN CELLS AND PLASMA OF FRESH
UNCITRATED AND CITRATED BLOOD.

	Uncitrated blood.		Citrated blood.	
	Per litre cell water.	Per litre plasma water.	Per litre cell water.	Per litre plasma water.
K m.eq.	146	4·5	146	3
Na m.eq.	17	155	17	188
HCO₃ m.eq.	22	26	20	17
Cl m.eq.	74	112	71·5	77
Non-penetrating anion m.eq. .	67	21·5	71·5	97

TABLE XII.—WATER AND BASE CONTENTS OF FRESH AND STORED CITRATED
BLOOD AT 2°.

	Fresh citrated blood.			Stored citrated blood.			
				Cells only at			
	Cells.	Plasma.	Na citrate solution.	1 week.	2 weeks.	4 weeks.	8 weeks.
Volume c.c. . .	100	110	52·5	103	119	131	135
Water per cent. .	70·5	93	..	71·5	75·5	77·5	78·0
K m.eq. per litre *	103	4	0	67·5	47·0	35·8	32·8
Na m.eq. per litre *	12	144	255	51·2	78·1	88·2	96·8

* Per litre final volume in the case of stored erythrocytes.

The increase in cell volume (Table XII) during storage is due to the
entry of water from the plasma. From these changes and the original
water contents of cells and plasma, the water contents of cells and
plasma during storage have been calculated. Similarly, the Na and K
contents in the plasma of stored blood have been calculated from the
total observed values for whole blood and the known values for Na
and K in stored cells. From all these data the concentrations of Na
and K in the cells and plasma of stored blood have been calculated
(Table XIII).

TABLE XIII.—CONCENTRATION OF K AND NA IN CELLS AND PLASMA OF BLOOD STORED WITH PLAIN CITRATE SOLUTION AT 2° (PH READINGS AT 17°).

	Cells.				Plasma.			
	pH.	K m.eq.	Na m.eq.	Na + K m.eq.	pH.	K m.eq.	Na m.eq.	Na + K m.eq.
Fresh uncitrated blood .	..	146	17	163	..	4·5	154	158·5
Fresh citrated blood .	7·54	146	17	163	7·57	3	189	192
Citrated blood at 1 week .	7·36	94	71	165	7·37	25	166	191
,, ,, ,, 2 weeks	..	62	104	166	..	38	156	194
,, ,, ,, 4 ,,	7·15	46	114	160	7·09	49	152	201
,, ,, ,, 8 ,,	..	42	124	166	..	53	146	199

Table XIII shows that cells originally more acid than plasma gradually become relatively less acid—although the acidity of both phases actually increases. It also shows that citration increases plasma base to a concentration which is maintained at a fairly constant level during two months' storage.

During the first two weeks of storage, the concentration of cell K gradually falls but still remains greater than the concentration of plasma K. The figures suggest that sometime in the second month of storage cell K falls below plasma K and that cation equilibrium is approached, when according to the Donnan equation

$$[K] \text{ cell}/[K] \text{ plasma} = [Na] \text{ cell}/[Na] \text{ plasma} = [K + Na] \text{ cell}/[K + Na] \text{ plasma}.$$

The observed ratios are 0·8, 0·85, and 0·83. The agreement is sufficiently close in view of all the experimental errors and of the possibility that the activities of Na and K are not necessarily the same in either cell or plasma.

Since during storage, the concentrations of base alters but little, it may seem paradoxical that both water and base should continue to enter the cells in increasing quantities. But this process is a direct result of citration which suddenly raises Na and hence total base in the plasma and at the same time adds a large amount of non-penetrating anion—citrate—to the extra-cellular phase. The resulting Donnan equilibrium requires that an excess of base remain in the citrated plasma, while an excess of penetrating anion be turned over to the cells, and that all this should take place without disturbing the osmotic equilibrium. The requirements can only be satisfied by the passage of water and anion, and of base in relatively low concentration from plasma to cell. Theoretically, it should be possible to calculate the equilibrium values for cell and plasma base, acid and water from the analyses of fresh blood, but this would involve the following approximations: (1) Osmotic pressure of hæmoglobin. (2) Osmotic pressure of cell phosphates. (3) Amounts, distribution, and osmotic pressure of

acid cell metabolites.   While even if information on all these data were available, the relevant volume of plasma is not known.   It is true that citrated blood contains 10 c.c. cells and 16·25 c.c. plasma, but when cells have settled, they are only in true equilibrium with the intercellular plasma and the plasma immediately above the settled mass.   It is not probable that diffusion alone without additional mixing will enable equilibrium to be established between the cell mass and the more distant parts of the supernatant plasma.   For these reasons, calculations beyond those necessary for Table XIII have not been attempted.

In stage 2, then, owing to the exces of non-penetrating anion it contains, the plasma is more acid than the cells and contains a higher concentration of base.   But owing to the large excess of base added at the moment of citration, equilibrium necessitates a flow of water and base into the cells.   Had the non-penetrating anion been increased without raising base, plasma would still have contained a higher concentration of base, but total cell base would not have increased and the concentration of cell base might have actually decreased, nor in this case would cell swelling have followed.

In spite of the fact that some cell swelling occurs in citrated blood, it would seem probable that the non-penetrating anion citrate has a definite function in storage besides that of preventing coagulation, in so far as it opposes a large and prolonged osmotic pressure to that exerted by cell hæmoglobin and phosphate, and in this way postpones osmotic hæmolysis.   In the absence of citrate, cell swelling would be much more rapid, and certainly, in non-citrated heparinised blood, hæmolysis is relatively quick.

This so-called second stage lasts for about two months after citration and during it chemical changes also occur, as a result of enzyme action. Inorganic phosphate is liberated from hydrolysable phosphate and in the process the base binding power of the phosphorus atom is increased [Lohmann, 1935].   Other organic phosphates are also degraded, but in this case the base binding power of the inorganic phosphate formed is, at physiological pH, less than that of the original phosphoric esters [Meyerhof and Lohmann, 1929].   The nett result is probably a small decrease in the non-penetrating anion of the erythrocytes.

Simultaneously, during the process of dephosphorylation, the organic portion of the esters are converted in several stages to lactic acid, and the resultant of the various reactions is to bring about an increased acidity of both blood phases.   If, however, the common practice is used of judging blood pH from plasma pH alone [Maizels and Whittaker, 1940 b; Mollison and Young, 1942], an exaggerated idea will be obtained of the changes in reaction, since some part of the fall in plasma pH results from physical changes due to the presence of the non-penetrating anion citrate in the plasma phase and is accompanied by a corresponding tendency to rise in cell pH, a tendency, however, which

is masked by the general increase in acidity due to lactic acid. However, an absolute increase in cell acidity does occur and as a result hæmoglobin⁻ is greatly decreased, so that its contribution to non-penetrating anion in the cells becomes unimportant. Against this may be set passage of citrate to the cells from the plasma, which by the end of a month has lost about 15 or 20 per cent. of its original content. The resultant of all these factors is that even after two months' storage plasma-base concentration still exceeds cell-base concentration, and it must be concluded that an excess of non-penetrating anion still persists in the extra-cellular phase.

Stage 3.—At the end of stage 2 about 25 per cent. of citrate has left the plasma and about 12 per cent. of cell phosphate has passed in the reverse direction from intact erythrocytes. Another moiety of phosphate has reached the plasma from cells which have hæmolysed—about 5 to 10 per cent. of the whole. At three months, intact cells have lost some 30 per cent. of their total phosphate and additional phosphate reaches the plasma by way of cells hæmolysed, some 30 per cent. of the erythrocytes have been destroyed in this way. It is probable that at some time in this stage non-penetrating anion will once more be in excess in the erythrocytes, and then the concentration of cell base will rise, while anion will fall and water will continue to flow into the cells.

Stage 4.—Hæmoglobin⁻ and plasma protein⁻, non-penetrating; all other ions penetrating. It is probable that this stage does not occur, complete hæmolysis having supervened. Theoretically, the relatively excessive osmotic pressure of hæmoglobin would attract water into the cells until hæmolysis was complete.

Weak Glucose Solutions.—The changes in blood stored with glucose-citrate solutions are qualitatively similar to those occurring with simple citrate solution. Table III shows that loss of K is similar in both solutions, but the presence of glucose delays the entry of sodium. Further, while in the case of simple citrate solution, cells and plasma approach equilibrium after a month and nearly attain it at two months, in the case of glucose-citrate solutions it is still fairly remote even after the longer period. The data in Table XIV have been calculated in the same way as those in Table XIII and it will be seen that after two months' storage the ratios of Cell/Plasma K, Na, and K + Na are respectively 0·94, 0·78, and 0·82.

The effect of glucose in conserving organic phosphate has already been noted. As a result inorganic phosphate in erythrocytes remains relatively low (Table III), while glucose is progressively converted to lactic acid. In the case of simple citrate solutions, the natural glucose of the blood is quickly used up and acid production is limited. But when glucose-citrate solutions are used, more potential acid is available and this may explain the relatively low pH of both plasma and cells in blood stored with this solution. The shift in pH is roughly equivalent

TABLE XIV.—CONCENTRATIONS OF K AND NA IN CELLS AND PLASMA OF BLOOD STORED WITH GLUCOSE CITRATE SOLUTION (PH READINGS AT 17°).

	Cells.				Plasma.			
	pH.	K m.eq.	Na m.eq.	Na + K m.eq.	pH.	K m.eq.	Na m.eq.	Na + K m.eq.
Fresh citrated blood .	7·54	146	17	.163	7·57	3	189	192
Citrated blood at 1 week .	7·27	93	70	˙163	7·22	26	165	191
,, ,, ,, 2 weeks	..	72	88	160	..	35	160	195
,, ,, ,, 4 ,,	7·00	53	101	154	6·90	43	160	203
,, ,, ,, 8 ,,	..	46	115	161	..	49	148	197

to 12 per cent. of the total buffer bound base after one week's storage and to about 30 per cent. after a month. This acidity may possibly have a bearing on the advisability of transfusing large volumes of glucose-citrate blood or plasma to patients with severe tissue anoxæmia from shock or hæmorrhage.

Another effect of the low pH of blood stored with glucose solutions is to bring hæmoglobin so much nearer its isoelectric point as to decrease its base binding power very considerably. For this reason the excess of non-penetrating anion in the plasma is relatively greater than in non-glucose preservatives, and as a corollary the concentration of cell base will be relatively low (Table XIV). Another factor in the low cell base may be decreased penetration of the cell membrane by cation at low pH.

The preservation action of glucose, however, cannot be explained only by the acid reaction it provokes, for the addition of acid without glucose does not preserve so effectively as glucose alone.

Rous-Turner's Solution.—This solution has four peculiarities: it is rich in glucose; its volume relative to that of the blood with which it is mixed is exceptionally large; it is relatively poor in base, and apart from its glucose content is hypotonic.

The immediate effect of adding this preservative is to dilute the plasma sixfold with a corresponding dilution of plasma chloride which falls to a much lower level than with any other diluent. This must result in a series of changes qualitatively like those described as occurring immediately after admixture of blood with simple citrate solution, but quantitatively much more marked. Thus chloride leaves the cells for the plasma, but owing to the slowness of movement of K and Na, it will have to pair with H^+ in its passage through the cell membrane. This implies an hydrolysis of neutral salts, a process which can only occur to a limited extent. The result is that a large excess of chloride must remain in the cells and, in accordance with the Donnan equation, a large excess of hydrogen ions must remain in the plasma.

Such an excess is in fact observed at an exceptionally early stage, and after only a week's storage it is found that the plasma is much more acid than the cells, and is indeed more acid than the plasma of either simple or glucose citrate systems.   The pH differences between cells and plasma are also much greater in the Rous-Turner system (Table XV).

TABLE XV.—CONCENTRATIONS OF K AND NA IN CELLS AND PLASMA OF BLOOD
    STORED WITH ROUS-TURNER'S SOLUTION (PH READINGS AT 17°).

	Cells.				Plasma.			
	pH.	K m.eq.	Na m.eq.	Na + K m.eq.	pH.	K m eq.	Na m.eq.	Na + K m.eq.
Fresh citrated blood .	..	146	17	163	..	1	118	119
Blood stored for 1 week .	7·52	72	42	114	7·15	8	119	127
,, ,, ,, 2 weeks .	7·32	49	52	101	7·07	12	117	129
,, ,, ,, 4 ,, .	7·10	38	48	86	6·91	13	120	133
,, ,, ,, 8 ,, .	..	23	64	87	..	15	114	129

During the days following the mixture of blood with Rous-Turner's solution, glucose diffuses into the cells so that the concentrations within and without tend to equalize and the osmotic pressures so exerted to cancel out.   The remaining osmotic pressure apart from that due to the original plasma contents is then exerted by sodium citrate equal to a content of 1·08 per cent. in the diluent.   Since an isotonic solution of sodium citrate contains 3·1 per cent., the Rous-Turner solution is, for practical purposes, very hypotonic and when mixed with blood causes the rapid initial swelling of cells which has been noted earlier, a swelling which would be a little greater were it not for the passage of chloride into the plasma with increase in the pH and corresponding decrease in the osmotic pressure of the cells.

Stage 2.—In this stage, external citrate acts as a non-penetrating anion, its content in the external plasma-diluent phase being initially about 90 m.eq.—rather more than in the simple and glucose citrate systems already described.   The excess of non-penetrating anion in the external phase imposes a concentration of base on the cells which must be much lower than that of the plasma.   But the base content of the unmixed Rous-Turner solution is already very low: 110 m.eq. compared with 255 m.eq. in simple and glucose citrate solutions before they are mixed with blood.   As a result the base content of the Rous-Turner plasma-diluent system is only 116 m.eq. as compared with 184 m.eq. in the two previous solutions.   For this reason cell-base concentration at equilibrium must of necessity be exceptionally low in the Rous-Turner system, and experimental findings show that this is the case (Table XV).   Further, owing to the dilution effect of the large volume

of preservative added, the low concentration of penetrating anion (Cl^- and HCO_3^-) required by the Donnan equation will already be realised to a greater or lesser extent in the plasma, and therefore water and base will not tend to pass from plasma to cells to the same extent as in the two previous systems where external base is particularly high; on the contrary, base passes in the opposite direction. For these reasons, the rather rapid initial rate of swelling observed with Rous-Turner's solution is not maintained: after one week's storage, swelling of cells with this solution is from 2 to 10 per cent. greater than with ordinary glucose-citrate; but after four weeks it is about the same.

In spite of the very low level to which cell K falls, it is still in excess of the external K (assuming this to be evenly distributed throughout the external medium) even after two months' storage. The cell/plasma ratios for K, Na, and K + Na are 1·53, 0·56, and 0·67; the figures indicate that equilibrium throughout the system is still remote and is exceptionally slow of attainment.

Apart from these ionic changes, there is little distinctive about blood stored with Rous-Turner's solution. Organic phosphate tends to disappear a little more slowly than with ordinary glucose-citrate solution, but hydrolysable phosphate does not last any longer. Clearly, then, these chemical changes do not seem to explain the greatly superior preservation afforded by Rous-Turner's solution. Other possible effects of the solution on sedimentation and "packing" have already been mentioned.

Acid Glucose-Citrate Solution.—Acidification of blood greatly decreases the base binding power of cell-hæmoglobin, while the citric acid added increases the non-penetrating anion in the plasma which is both

TABLE XVI.—CONCENTRATIONS OF K AND NA IN CELLS AND PLASMA OF BLOOD STORED WITH ACID GLUCOSE-CITRATE SOLUTION AT 2° (PH READINGS AT 17°).

	Cells.				Plasma.			
	pH.	K m.eq.	Na m.eq.	Na + K m.eq.	pH.	K m.eq.	Na m.eq.	Na + K m.eq.
Fresh citrated blood .	..	146	17	163	..	3	189	192
Citrated blood at 1 week .	7·13	98	52	150	7·07	22	176	198
„ „ „ 2 weeks	..	77	67	144	..	31	171	202
„ „ „ 4 „	6·97	60	77	137	6·82	39	173	212
„ „ „ 8 „	..	47	96	153	..	48	158	206

relatively and absolutely greater than with simple and glucose citrate solutions. For this reason, cell base tends to be lower and plasma base higher than with less acid systems, and during storage cell-base content and concentration may both be lower than they were in the original unstored cells (Table XVI). At the same time the acid delays the

penetration of cation into the erythrocytes [Mond, 1928] and this is shown by the slowness with which K leaves the cells during storage and the slowness with which Na enters. It is also shown by the absence of true equilibrium in the blood even after two months' storage, when the concentration of K is still high in the cells, while that of Na remains high in the plasma and the cell/plasma rations of K, Na, and K + Na are respectively 0·98, 0·61, and 0·75 (Table XVI).

The effect of acid on dephosphorylation has already been described. There is a conservation of hydrolysable phosphate with a breakdown of other organic phosphate so rapid as to overshadow the inhibiting effect of glucose almost entirely.

PHOSPHATE AND POTASSIUM IN STORED ERYTHROCYTES.

In this section the relation of K to organic phosphate has been considered. No correlation was observed, but certain observations on cell phosphate are set out below. The observations were originally made during the course of an investigation to determine if conservation of phosphoric esters favoured blood preservation.

Phosphate in Erythrocytes Stored at 2°.

pH and Dephosphorylation.—It is well known that a slight degree of acidity is optimal for the action of cell phosphates [Lawaczec, 1924; Rapoport and Guest, 1939]. This is shown in Table XVII, where inorganic phosphate liberated in cells stored with glucose-citrate solutions for one and two weeks is recorded.

TABLE XVII.—EFFECTS OF pH ON INORGANIC PHOSPHATE LIBERATED IN ERYTHROCYTES OF BLOOD STORED AT 2° WITH GLUCOSE-CITRATE SOLUTIONS FOR ONE AND TWO WEEKS (pH READINGS AT 17°).

	1 week.	2 weeks.	1 week.	2 weeks.	1 week.	2 weeks.	1 week.	2 weeks.
Cell pH . . .	7·77	..	7·27	7·01	7·03	6·92	6·85	..
Inorganic P mg. per cent.	1·34	1·52	1·42	6·72	4·93	23·5	23·1	32·0

The initial inorganic phosphate in the cells of this experiment was 1·68 mg. per cent., and as after a week's storage inorganic phosphate in the cells of the most alkaline system had declined to 1·34 mg., it must be assumed that at high pH synthesis of organic phosphate esters exceeds breakdown—a process previously described by Martland, Hansman, and Robison [1924] and illustrated again later in this paper.

If no alkali is added to citrated blood, the pH after a week's storage is about 7·2 and inorganic phosphate shows very little change, *some-*

times rising slightly and at others falling. A small decrease is not at all uncommon and may account for the slight drop in the inorganic phosphate of the plasma observed early in storage by Aylward, Mainwaring, and Wilkinson [1940]: for if cell and plasma inorganic phosphate are in equilibrium, and then as a result of synthesis cell inorganic phosphate falls, then inorganic phosphate should pass from plasma to cells. Such a passage might explain the very slight increase in total cell phosphate sometimes observed during the earlier stages of storage.

In the case of acid systems, Table XVII shows a rapid accumulation of inorganic phosphate at a very early stage of storage. In neutral or alkaline solutions, this does not occur until acid products of metabolism have changed the reaction of the erythrocytes and so imparted a certain momentum to phosphate breakdown. In the case of the most alkaline systems this may take a long time and then, even after a month, little change in organic phosphate occurs. On the other hand, in the absence of glucose, breakdown of organic phosphate is rapid even in slightly alkaline systems.

Hydrolysable Phosphate.—Table XVIII shows that hydrolysable phosphate gradually decreases during storage. Breakdown is most rapid at about neutrality, is less active in alkaline solutions and still less active at acid reactions.

TABLE XVIII.—EFFECTS OF pH ON THE BREAKDOWN OF HYDROLYSABLE PHOSPHATE IN ERYTHROCYTES STORED FOR TWO WEEKS WITH GLUCOSE-CITRATE SOLUTION AT 2°. (INITIAL VALUE 11·1 MG. PER CENT. pH READINGS AT 17°.)

pH	7·38 +	7·38	7·01	6·92	6·92 −
Hydrolysable phosphate mg. per cent.	6·9	6·0	5·2	7·5	7·6

Hydrolysable phosphate persists longest if both glucose and acid are present. If acid alone be added to the system, hydrolysable phosphate survives less well, but the difference is small and within the range of experimental error. But if neither glucose nor acid are present breakdown of hydrolysable phosphate is rapid (Table XIX).

Some light may be thrown on the preceding findings by a brief consideration of the possible course of the phosphate cycle in erythrocytes. Investigation of the problem in muscle and tissue extracts has followed the direction indicated by the theories of Embden, Meyerhof, Parnas, and others. A review of the extensive literature on this subject is given by Lipmann [1941]. The phosphate cycle in erythrocytes, however, may well be different from that occurring in muscle. Thus, quantitatively, the chief phosphoric ester in erythrocytes is 1-3-diphosphoglycerate [Greenwald, 1925], yet this substance plays no part in the muscle cycle, and although it may be concerned in the erythrocyte

TABLE XIX.—EFFECT OF DILUENT ON THE DISAPPEARANCE OF HYDROLYSABLE PHOSPHATE IN ERYTHROCYTES STORED FOR TWO WEEKS AT 2°. (INITIAL VALUE 10·7 MG. PER CENT.)

Diluent . Hydrolysable P. mg. per cent.	Glucose-Citrate. 7·0	Plain-Citrate. 3·4	Dextrin-Citrate. 2·9	Sucrose-Citrate. 3·4

cycle, its action is obscure. The presence of adenosine triphosphate in both blood and muscle cells suggests, however, that certain processes are common to both. It is probable that in erythrocytes adenosine triphosphate reverts to adenylic acid, at the same time donating phosphate to hexose derivatives and converting these to hexose diphosphate. The latter is converted by several intermediate stages to phosphopyruvate. Phosphopyruvate donates phosphate to adenylic acid, which is thereby restored to adenosine triphosphate, while the pyruvate is converted to lactate. In this way the cycle is complete, the energy for its maintenance being derived from the breakdown of glucose. It should perhaps be mentioned that in the scheme set out no mention has been made of other phosphorylating systems.

In the circulation, means for supplying glucose and removing lactic acid are continuously available, but during storage the supply of glucose may be limited and acid products accumulate. If no extra glucose is added, sugar naturally present in the blood is soon used up and the cycle is interrupted, for the resynthesis of phosphoric esters is no longer possible and those remaining are broken down by phosphatase. Donation and acceptance of phosphate by the adenylic acid system comes to an end, and the end products of phosphate metabolism are now lactic acid and inorganic phosphate. At this stage the tendency of enzyme action to convert adenosine triphosphate into adenylic acid and inorganic phosphate is no longer balanced by a rephosphorylation in the reverse direction, since primary phosphorylation of adenylic acid by inorganic phosphate does not occur. There results a progressive conversion of adenosine triphosphate to adenylic acid which is manifested by a decline in hydrolysable phosphate (Table XIX).

If the glucose content of the citrated blood is high, the phosphorolysis cycle is maintained for a longer time, but as lactic acid accumulates and the pH of the system falls, the rate of breakdown exceeds that of synthesis and the balance of the system is lost. Slower synthesis obstructs the donation of phosphate by adenosine triphosphate, and here again, as in systems containing no added glucose, inorganic phosphate accumulates and hydrolysable phosphate ultimately falls (Table XIX).

The effects of pH on hydrolysable phosphate seem paradoxical in

that a rapid accumulation of inorganic phosphate occurs, but hydrolysable phosphate disappears more slowly than in slightly alkaline systems. It would seem that adenosine triphosphatase is most active at slightly alkaline reactions, less active at more alkaline reactions, and much less active at acid reactions. But even in the presence of acids some breakdown of adenosine triphosphate does occur (Table XVIII), although more than 50 per cent. may survive after a month's storage.

In view of the effects of fluoride on the phosphorolysis cycle, its action on stored blood was examined. Added to a content of 0·0015 M, it produced the following effects: (1) Fall of pH during storage is delayed. (2) Spontaneous hæmolysis is greatly increased possibly owing to greater activity of hæmolysin at high pH. (3) Breakdown of slowly hydrolysable phosphoric esters is delayed. (4) Disappearance of hydrolysable phosphate is accelerated (Table XX).

TABLE XX.—EFFECTS OF FLUORIDE ON BLOOD STORED FOR TWO WEEKS AT 2° (pH OBSERVATIONS AT 17°).

Solution.	Spontaneous hæmolysis, per cent.	pH cells.	Hydrolysable phosphate, mg. per cent.	Inorganic phosphate, mg. per cent.
Glucose-citrate . .	0·14	7·10	4·9	10·1
Glucose-citrate-fluoride	1·9	7·38	1·5	2·1

Fluoride acts on the phosphorolysis cycle by preventing the formation of phosphopyruvate [Lohmann and Meyerhof, 1934] and so the production of inorganic phosphate and lactic acid is delayed. At the same time, adenosine triphosphate continues to donate phosphate and revert to adenylic acid, but in the absence of phosphopyruvate, adenylic acid cannot be re-phosphorylated—hence the decrease in hydrolysable phosphate recorded in Table XX.

Permeability of Stored Erythrocytes to Inorganic Phosphate.

In blood stored with or without glucose, an increase of the inorganic phosphate within the cells ultimately occurs, but in spite of this, if allowance is made for concentration of plasma by passage of water into cells and for direct increase of plasma phosphate by hæmolysis of cells, the nett increase in the inorganic phosphate of the plasma is very small and roughly balances phosphate lost from the erythrocytes as determined by direct estimation of cell contents (Table XXI).

In the above experiments, percentage hæmolysis, phosphate contents, etc., have been corrected for changes in cell and plasma volume. In order to assess the nett losses and gains a further allowance must

TABLE XXI.—NETT LOSS OF PHOSPHATE BY ERYTHROCYTES AND NETT GAIN BY PLASMA OF BLOOD STORED WITH SIMPLE CITRATE SOLUTION AT 2°.

Experiment.	Storage weeks.	CELL PHOSPHATE.					PLASMA INORGANIC PHOSPHATE.			
		Spontaneous hæmolysis, per cent.	Total original, mg. per cent.	Total final, mg. per cent.	Nett loss, mg. per cent.	Inorganic, mg. per cent.	Original, mg. per cent.	Gained by cell hæmolysis, mg. per cent.	Final, mg. per cent.	Nett gain by diffusion from cells, mg. per cent.
1	2	0·86	55·0	53·1	1·9	31·3	2·5	0·5	3·6	0·6
2	4	3·3	53·4	50·2	3·2	45·8	2·3	1·7	6·4	2·4

be made for the relative volume of cells and plasma. Thus in experiment 1, 100 c.c. original blood contained 36 c.c. of cells and 64 c.c. of plasma. Hence the absolute loss of phosphate by cells was $1·9 \times 36/100$ or 0·7 mg., while the gain by plasma was $0·6 \times 64/100$ or 0·4 mg. In experiment 2 the absolute cell loss was 1·2 mg. and the plasma gain 1·5 mg.

It follows therefore that, although inorganic phosphate reaches a high level in stored cells, very little escapes into the plasma even after five weeks.

Nevertheless, although egress of phosphate seems difficult, ingress is relatively rapid for if inorganic phosphate at pH 7·4 is added to blood an increase of total and inorganic phosphate within the erythrocytes occurs in a few days (Table XXII).

TABLE XXII.—INCREASE OF CELL PHOSPHATE ON THE ADDITION OF SODIUM PHOSPHATE TO BLOOD STORED WITH SIMPLE CITRATE SOLUTION FOR ELEVEN DAYS AT 2°.

PLASMA PHOSPHATE. Original + added referred to initial plasma volume.	CELL PHOSPHATE. Original + gained referred to initial cell volume.		CONCENTRATION OF INORGANIC PHOSPHATE in water of	
Inorganic P, mg. per cent.	Total P, mg. per cent.	Inorganic P, mg. per cent.	Plasma, mM.	Cells, mM.
3·22	51·8	33·7	1·04	13·2
10·2	53·8	35·4	3·77	13·6
16·9	56·4	36·9	6·24	14·7
29·4	61·0	41·2	10·9	16·4

In columns 1, 2, and 3 of Table XXII, values are referred to the initial cell and plasma volume and figures represent absolute changes in contents. Thus in column 2, original total P was 51·3 mg. per cent.,

that a rapid accumulation of inorganic phosphate occurs, but hydrolysable phosphate disappears more slowly than in slightly alkaline systems.   It would seem that adenosine triphosphatase is most active at slightly alkaline reactions, less active at more alkaline reactions, and much less active at acid reactions.   But even in the presence of acids some breakdown of adenosine triphosphate does occur (Table XVIII), although more than 50 per cent. may survive after a month's storage.

In view of the effects of fluoride on the phosphorolysis cycle, its action on stored blood was examined.   Added to a content of 0·0015 M, it produced the following effects: (1) Fall of pH during storage is delayed.   (2) Spontaneous hæmolysis is greatly increased possibly owing to greater activity of hæmolysin at high pH.   (3) Breakdown of slowly hydrolysable phosphoric esters is delayed.   (4) Disappearance of hydrolysable phosphate is accelerated (Table XX).

TABLE XX.—EFFECTS OF FLUORIDE ON BLOOD STORED FOR TWO WEEKS AT 2° (pH OBSERVATIONS AT 17°).

Solution.	Spontaneous hæmolysis, per cent.	pH cells.	Hydrolysable phosphate, mg. per cent.	Inorganic phosphate, mg. per cent.
Glucose-citrate . .	0·14	7·10	4·9	10·1
Glucose-citrate-fluoride	1·9	7·38	1·5	2·1

Fluoride acts on the phosphorolysis cycle by preventing the formation of phosphopyruvate [Lohmann and Meyerhof, 1934] and so the production of inorganic phosphate and lactic acid is delayed.   At the same time, adenosine triphosphate continues to donate phosphate and revert to adenylic acid, but in the absence of phosphopyruvate, adenylic acid cannot be re-phosphorylated—hence the decrease in hydrolysable phosphate recorded in Table XX.

Permeability of Stored Erythrocytes to Inorganic Phosphate.

In blood stored with or without glucose, an increase of the inorganic phosphate within the cells ultimately occurs, but in spite of this, if allowance is made for concentration of plasma by passage of water into cells and for direct increase of plasma phosphate by hæmolysis of cells, the nett increase in the inorganic phosphate of the plasma is very small and roughly balances phosphate lost from the erythrocytes as determined by direct estimation of cell contents (Table XXI).

In the above experiments, percentage hæmolysis, phosphate contents, etc., have been corrected for changes in cell and plasma volume. In order to assess the nett losses and gains a further allowance must

It has been shown that erythrocytes rendered chemically abnormal by loss of K and gain of Na during storage at 2° are "reconditioned" in the recipient's circulation after transfusion. By analogy it would appear likely that normal erythrocytes are not impermeable to base, but are continually tending to lose K and gain Na and that the resulting changes are continually reversed by some counter-mechanism. Such a process might take place through the influence of some external factor: it might occur, for example, as the erythrocytes pass through the liver or spleen or other organ. Alternatively, the mechanism for "reconditioning" may exist in the cells themselves. Muscle cells present a similar paradoxical distribution of potassium, and since they are fixed and unable to wander in and out of organs, the process for their reconditioning must exist locally, and suggests that a similar local process may occur in erythrocytes.

Various theories have been advanced to explain the high K content of muscle. Some of these make assumptions that do not conform with the facts. This is the case with the theory of Conway and Boyle [1939] which assumes that muscle cells are permeated by K and not by Na. Dean [1941] suggests that a pumping mechanism exists in muscle which ejects Na and so determines a compensatory entry of K or alternatively that K is pumped into the muscle cell with a secondary expulsion of Na. He defines the physical conditions under which such a pump would produce a cation distribution conforming with experimental data, but is unable to suggest what motive force drives the pump.

Steinbach [1940] has shown that K can readily be washed out of excised frog's muscle in K-free Ringer's solution, and that it will again concentrate in the muscle against the concentration gradient if the muscle is placed in Ringer containing quite a low content of K. He suggests that if "the apparent dissociation of some organic potassium compound within the cell was less than that of the corresponding sodium compound and if the organic anion was indiffusible, then distribution ratios of total sodium and potassium within and without probably would be altered in the correct direction; that is, toward a high cellular content of potassium." This explanation would hardly apply to the erythrocyte, however, for here chloride and bicarbonate are presumably capable of binding about 65 m.eq. base. If all cell Na were so bound, there would still remain 40 m.eq. unbound by Na and therefore of necessity bound by K. From this it follows that K combined with chloride and bicarbonate within erythrocytes is alone at least eight times greater than external K.

Since, however, an inequality of cation distribution does exist, a dynamic "reconditioning" process is implied and the energy required for such a process might well derive from the active phosphate cycle which is such an important part of cell metabolism. No correlation,

while after 11 days storage it was 46·0 mg. per cent. But as the cells had swollen by 12·5 per cent., the actual change in total phosphate was from 51·3 to 46 × 112·5 or 51·8 mg. per cent. In fact the slight increase is within the range of experimental error and the figures should be taken to mean that no change in cell phosphate had occurred. The data in columns 4 and 5 represent true concentrations.

The first line of the table shows the control observation. Here, no phosphate was added, inorganic phosphate in cells rises to 33·7 mg. per cent., but total cell phosphate remains practically unaltered, indicating that no phosphate has diffused outwards. On the other hand an increase of external inorganic phosphate to 29·4 mg. causes a marked increase in the cell, showing that inorganic phosphate has passed inwards from the plasma. In the table, the data for concentrations make it appear that phosphate has entered the cells against the concentration gradient, but it is probable that entry was effected at an early stage before inorganic phosphate had accumulated as a result of dephosphorylation of cell esters, and when the plasma concentration of inorganic phosphate was many times greater than that of the cells. Once it has entered, however, passage in the reverse direction seems to be hindered.

According to Halpern [1936] no phosphate moves across the erythrocyte membrane at low temperatures, but the period of observation only covered a few hours and not days as in the present investigation. At 37° Halpern finds that phosphate moves fairly freely in and out of the cells, the direction being determined by metabolic activity. At 2°, however, exit of inorganic phosphate from cells is very slow, whether dephosphorylation is accelerated by the presence of acids or the absence of sugar, or whether it is delayed by alkali. A tentative suggestion is made that at 2° the erythrocyte membrane opposes a resistance to the passage of phosphate and that the resistance is considerably greater to passage of inorganic phosphate outwards.

Potassium in Erythrocytes.

In freshly shed blood cell K is high and Na is low, while in the plasma it is the Na which is high and the K low. During storage the respective concentration gradients become effective and cell K falls while Na rises. In the circulating blood, however, the anomalous distribution is maintained for long periods, and although in certain pathological states K may leave the erythrocytes, under most circumstances the cation contents of cell and plasma remain constant. Peters and Van Slyke [1937] remark that no explanation exists for the inequalities of distribution, which must, however, depend on some restraining factor in the cell membrane or on inherent characteristics of the cellular and extra-cellular media.

TABLE XXIV.—SYNTHESIS OF PHOSPHORIC ESTERS AND LOSS OF POTASSIUM
FROM ERYTHROCYTES STORED AT 2°.

Diluent.	Storage weeks.	Hydro-lysable phosphate, mg. per cent.	Other organic phosphate, mg. per cent.	Inorganic phosphate, mg. per cent.	K m.eq.
None (initial values) . . .	0	10·7	41·2	1·83	104
Alkaline glucose citrate (control) .	2	5·4	45·3	2·20	54
Alkaline glucose phosphate citrate	2	5·7	49·8	6·42	52
Acid glucose citrate (control) .	2	6·1	20·6	26·0	68
Acid glucose fluoride citrate .	2	1·5	42·2	9·3	72

that demonstrated by Steinbach [1940] for frog's muscle *in vitro*, has not been shown in the case of human erythrocytes.

It may well be that the alkaline phosphate cycle at 2° differs from the normal physiological process and this will certainly be the case if fluoride is added. In addition there are other activities in erythrocytes which might be associated with phosphate retention. Finally, it is possible that activities present at 37° are restrained at lower temperatures.

CONCLUSIONS.

1. Numerous chemical and physical changes affect human erythrocytes during storage. The physical changes include loss of K, gain of Na, and increase of Na + K. Increase in Na + K taken up by the cells determines secondary changes like cell swelling and increased fragility. Chemical changes include degeneration of the erythrocyte membrane *in vitro*, with consequent hæmolysis. Hydrolysis of phosphoric esters also occurs with decrease in organic phosphate affecting both the easily and not easily hydrolysable fractions. Inorganic phosphate shows a corresponding increase. The addition of glucose delays most of these changes, including the breakdown of easily hydrolysable and total phosphate. Acids delay hæmolysis *in vitro*, preserve hydrolysable phosphate, but accelerate the breakdown of other organic phosphates. They also delay cation exchange across the cell membrane.

2. Preservatives have different and uncorrelated effects in delaying or accelerating these various physical and chemical changes. In general, the slightness of a physical or chemical change cannot be used as a measure of the excellence of a diluent as a preservative, since there is no correlation between these tests and the available data for cell survival after transfusion, as set out by Mollison and Young [1942].

Spontaneous hæmolysis, however, has a definite negative value, for its rapid development *in vitro* is associated with poor survival of the

however, has been found between the level of erythrocyte K and the amounts of the various phosphate fractions. Thus in glucose-citrate systems, hydrolysable and total organic phosphate are relatively high while inorganic phosphate is low, while in citrate systems without glucose these findings are reversed, yet in both K loss from cells over a period of a month is about the same. It is true that acid systems show both a high inorganic and hydrolysable phosphate and that in these loss of K is slow, but here the influence of pH on K retention is probably exerted directly, through decreased permeability of the erythrocyte

TABLE XXIII.—SODIUM, POTASSIUM, AND PHOSPHATE FRACTIONS OF ERYTHROCYTES STORED AT 2°.

Diluent.	Storage weeks.	Hydro-lysable organic phosphate, mg. per cent.	Other organic phosphate, mg. per cent.	Inorganic phosphate, mg. per cent.	K m.eq.	Na m.eq.
Initial values . . .	0	10·2	42·2	1·2	102	14
Very acid glucose-citrate .	2	9·4	10·1	30·8	82	42
Acid glucose-citrate . .	2	8·5	18·8	24·9	71	47
Standard glucose-citrate .	2	5·0	37·9	9·8	62	61
Alkaline glucose-citrate .	2	6·9	41·5	1·3	56	87
Plain citrate . . .	2	1·4	20·4	31·3	62	76

membrane and not indirectly through its action on the phosphate cycle (Table XXIII).

An attempt was made to determine if retention of K by erythrocytes might be favoured if synthesis of phosphoric esters were encouraged. Blood was mixed with the following diluents: (1) Alkaline citrate with added glucose and phosphate; alkaline glucose citrate being used as a control. (2) Acid fluoride glucose-citrate solution, with acid citrate as control (Table XXIV).

Direct phosphorylation of glucose by inorganic phosphate does not occur. Nevertheless at high pH glucose and inorganic phosphate in erythrocytes combine to form organic phosphate, by an indirect process of phosphorylation, comparable perhaps to that described by Meyerhof [1941]. In spite of the synthesis of organic phosphate, however, loss of potassium has not been prevented.

With the acid fluoride systems, although increase of slowly hydrolysable phosphate is slight and occurs largely at the expense of hydrolysable phosphate, the phosphate composition contrasts greatly with that found in erythrocytes stored in fluoride-free acid systems, where organic phosphate is greatly reduced. In spite of this, the potassium contents of cells stored in either system are not dissimilar and at present a movement of K against the concentration gradient, comparable to

lysed plasma, and a lower layer of settled cells. It is sometimes convenient for other reasons to remove part of the supernatant plasma (*e.g.* to accommodate more cells in a centrifuge tube), and provided no hæmolysed plasma is removed this will not affect the estimation of spontaneous hæmolysis. It is necessary, however, that volume of residual plasma shall be not less than half that of the cells. Otherwise, even though no hæmolysed plasma is removed, serious errors are introduced.

Fragility.—1 volume of blood was mixed with 20 volumes of hypotonic saline and the percentage of cells hæmolysed estimated by comparison with suitable standards.

Sodium and potassium were estimated on centrifuged cells dried at 105° and ashed at just below 500°. Sodium was estimated by Salit's [1932] method, modified to prevent precipitation of potassium [Maizels and Paterson, 1940]. Potassium was measured by King, Haslewood, Delory, and Beall's modification [1942] of Jacobs and Hoffman's method [1931]. In addition, it was found convenient to hasten the solution of the potassium cobalti-nitrite by boiling it with a solution of acid sodium phosphate (0·3 per cent.) instead of with water.

Phosphate was estimated by Briggs' method [1924]. Hydrolysable phosphate + Inorganic phosphate was measured by boiling the trichloracetic acid filtrate with sulphuric acid added to normal strength. At first the period of boiling was ten minutes; later this was shortened to seven minutes. The longer time made no qualitative and only very slight quantitative difference to the results. Hydrolysable phosphate is the difference between the value so obtained and the value for inorganic phosphate, and the error is the sum of the experimental errors in the two estimations. If hydrolysable phosphate is between 7 and 10 mg. per cent., the error is about ± 3 per cent.; if it is in the neighbourhood of 1 mg. per cent., the error rises to about ± 15 per cent.

The estimation of total acid soluble phosphate was preceded by wet-ashing the filtrate with sulphuric acid and hydrogen peroxide (100 volumes). The peroxide gave a strong reaction for phosphate and had to be treated with magnesium oxide and then filtered before use. The presence of residual magnesium in the reagent did not seem to hinder its action, but a slightly acid reaction was needed to delay decomposition of the peroxide.

Citrate [Pucher, Sherman, and Vickery, 1936].—pH was usually measured in the glass electrode at 17°. The pH of a part of the settled cells was first estimated; this represents the pH of intercellular plasma at equilibrium with cells. The residue of cells were centrifuged, the supernatant plasma removed and the packed erythrocytes frozen and thawed three times. So long as some of the cells were hæmolysed, the completeness of the process is not important. Loss of carbon dioxide was prevented as far as possible.

remaining unhæmolysed cells after transfusion. The reverse is not quite true for polysaccharides for they prevent hæmolysis *in vitro,* but in the case of dextrin at least do not aid survival after transfusion. Apart from this, correlation between hæmolysis during storage and survival after transfusion is close, though other exceptions may emerge. A similar negative value may, perhaps attach to the disappearance of hydrolysable phosphate: diluents causing rapid disappearance are associated with poor survival *in vivo,* but persistence of hydrolysable phosphate in stored erythrocytes is not necessarily associated with good survival after transfusion.

3. Citrate besides preventing coagulation of the blood has another function: it adds to the plasma a large amount of non-penetrating anion, which is now in excess of, and in osmotic opposition to, the non-penetrating anion present in erythrocytes. This tends to delay entry of water into the cells and to limit the consequent swelling. It also brings about an increase in cation concentration and acidity of the plasma, which, in contrast to the findings in uncitrated blood, now exceed *the cation and hydrogen ion concentrations in the cells.*

4. The permeability of phosphate at $2°$ is peculiar in that penetration of phosphate from without inwards takes place much more readily than passage in the reverse direction.

5. No correlation has been found at $2°$ between the rate of hydrolysis of phosphoric esters in stored erythrocytes and the speed with which potassium escapes from the cells.

NOTE ON METHODS.

Blood to be analysed was gently mixed and centrifuged in hæmoglobin comparator tubes for 30 minutes at 3000 r.p.m. Cell and plasma volume were measured and supernatant plasma removed. The tubes were again centrifuged for 15 minutes and residual plasma together with the surface layer of packed erythrocytes removed.

Change in Volume.—1 c.c. packed erythrocytes were added to a 250 c.c. flask with a "wash-out" pipette and made up to volume. The solution was compared in a colorimeter with a similar dilution of the centrifuged original cells, which had been kept undiluted at $2°$. Percentage volume = Reading of unknown + 100/Reading of standard.

Spontaneous Hæmolysis.—The method for this is described elsewhere [Maizels and Whittaker, 1939]. Stored blood settles in three layers: an upper clear unhæmolysed layer of plasma, a middle layer of hæmo-

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The method presents, among others, two sources of error: (1) Blood was stored at 2° and cells and plasma were separated while still cold, while pH was measured at 17°. There is a considerable temperature coefficient involved which is probably not the same for cells and plasma. (2) The pH of the hæmolysed cell mass is not necessarily the same as that of the interior of the intact cells. For these reasons pH values have no absolute, but only relative values. A consistent technique was followed as far as possible, but the data should be regarded merely as indicating the direction in which pH is tending to move.

In the experiments on pH and the action of hæmolysins, the reaction was measured with indicator dyes.

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CITRIC ACID-SODIUM CITRATE-GLUCOSE MIXTURES FOR BLOOD STORAGE. By J. F. LOUTIT, P. L. MOLLISON, and I. MAUREEN YOUNG,[1] with the technical assistance of E. J. LUCAS.

A REPORT TO THE MEDICAL RESEARCH COUNCIL FROM THE SOUTH-WEST LONDON BLOOD SUPPLY DEPOT.

INTRODUCTION.

FOR blood transfusion purposes trisodium citrate has long been the most widely used anticoagulant; for blood storage the addition of glucose to the citrated blood as a preservative is now accepted as invaluable—not only is hæmolysis retarded but the survival *in vivo* of the erythrocytes after transfusion is greatly prolonged. This mixture of trisodium citrate and glucose has, however, one great disadvantage. As the citrate has an alkaline reaction (pH 8·5–9) the glucose is caramelised when the two are autoclaved together. All workers have taken care to avoid this brown coloration, although the nature of the objection has not been stated. The universal practice has been to sterilise the two solutions separately and then to mix them with aseptic precautions. This procedure is most time-consuming and increases the risk of bacterial contamination.

The present standard anticoagulant preservative mixture in general use in England since 1940 is: 100 c.c. of 3 per cent. trisodium citrate + 20 c.c. of 15 per cent. glucose (or 10 c.c. of 30 per cent. glucose) for admixture with 420 c.c. of blood (Medical Research Council War Memorandum, No. 1), and is hereafter called the M.R.C. solution. This investigation was carried out, firstly, to determine whether this solution could be modified by the addition of citric acid so that the formation of caramel on autoclaving would be reduced or abolished, and, secondly, to see what effect this modification would have on the preservative powers of the solution. Before recommending any new solution for use as a blood preservative it is necessary to establish that red cells stored in the solution survive well in the recipient after transfusion. It is not sufficient to show that the solution retards various biochemical and

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citrate were also tried.   To 100 c.c. of each solution 10 c.c. of a 30 per cent. solution of anhydrous glucose were added.   All chemicals used were of analytical reagent standard.

To determine approximately how much glucose had been lost the total reducing substances left after autoclaving were estimated as "glucose" by Hanes' [1929] method.

No attempt was made to estimate the caramel as such because it is known that one part of this complex in 10,000 parts of water gives a very dark brown solution, and, judging from the colour of our solutions, even smaller quantities must have been present.

A sodium citrate-citrate acid-glucose solution (100 c.c. of a 0·64 per cent. trisodium citrate solution in 0·36 per cent. citric acid + 20 c.c. of 30 per cent. glucose) devised by Murray [1943] was also investigated (No. 8, Table I).

<div align="center">RESULTS.</div>

From Table I it is seen that when the M.R.C. diluent was autoclaved complete it became the colour of very dark sherry, but as the pH was lowered smaller amounts of caramel were produced till *in this series* at a pH below 4·7 the solutions were colourless.   (It may be noted that similar colours were produced when the solutions were autoclaved in a steam-heated, steam-jacketed autoclave at 15 lb. pressure for 30 minutes.   A recording thermometer registered 122° C.   A hundred minutes elapsed before the temperature fell to 84° C., when the autoclave was opened.)

The destruction of glucose was not great in any of the solutions investigated.   Solution No. 1 was found to have lost 13·5 per cent. of the "glucose" originally present and at the same time the pH fell from 8·5–9 to 5·8; diluent No. 4 lost 2·5 per cent. "glucose" and the more acid diluents even smaller quantities.

(b) *Retardation of Spontaneous Hæmolysis.*—Blood was stored in each of the solutions 2–8 and in the standard M.R.C. solution at 3–7° C. for 4 weeks, at the end of which time the bloods were well mixed and centrifuged.   The amounts of free hæmoglobin in the plasmas were then compared.

By this crude method it was noted that the spontaneous hæmolysis in solutions nos. 2, 3, 4, 6, and 8 was less than in the M.R.C. diluent, in solution no. 5 about the same and in solution no. 7 it appeared to be somewhat greater.

<div align="center">DISCUSSION.</div>

Heating glucose for prolonged periods at high temperatures favours the formation of caramel.   With autoclaves that can be rapidly heated and exhausted of air it should be possible to ensure sterilisation of the contents with shorter periods of heating or at lower temperatures than

physical changes during storage, since the latter changes are sometimes fallacious indicators of preservation as judged by the subsequent survival *in vivo* of the red cells [Maizels, 1941 *a*; Mollison and Young, 1941 and 1942]. The procedure involved in the estimation of survival is so lengthy, however, that, while it was considered indispensable for the main investigation, the criterion adopted in the preliminary selection of solutions for trial was the retardation of spontaneous hæmolysis. This test enables unsatisfactory solutions to be rejected since preservatives which do not retard hæmolysis cannot be regarded as efficient.

PRELIMINARY SELECTION OF SOLUTIONS.

(a) *Prevention of Caramelisation.*—The degree of caramelisation of glucose depends, among many factors, on the temperature to which it is heated, the time for which it is heated, the pH of the solution and the concentration of glucose. With the autoclaves available, it was not considered desirable to reduce either the working pressure or the time [Spooner and Turnbull, 1942].

Muether and Andrews [1941] claimed that they could prevent caramelisation of their glucose citrate solution by autoclaving it at 15 lb. pressure for 20 minutes with a phosphate buffer. Evans, Thorley, and McLeod [1942], using the M.R.C. sodium citrate-glucose solution, maintained that saturation with carbon dioxide and autoclaving at 15 lb. pressure for 30 minutes with the caps of the bottles screwed tight prevented coloration.

A preliminary trial showed that neither of these methods was satisfactory with our autoclaves, because the resulting pH was not low enough. A simple way of decreasing the pH of the citrate-glucose mixture seemed to be to replace varying quantities of the sodium citrate with citric acid.

METHODS.

The autoclaves used were gas-heated, air-jacketed, vertical autoclaves, 1 ft. 9 in. in diameter by 3 ft. 5 in. high, the standard autoclaves issued to the War Emergency Blood Transfusion Services. With each run, a period of 30 minutes free steaming with valve open, to ensure as complete air exhaustion as possible, was followed by 30 minutes sterilisation at 20 lb. pressure. A further 30–40 minutes ensued before the pressure fell to atmospheric, at which point the valve was opened and the autoclave emptied.

All bottles used, which were the standard M.R.C. 1-pint bottles, were autoclaved with the caps screwed down tight.

3 per cent. dihydric trisodium citrate and 3 per cent. monohydric citric acid were mixed in the proportions shown in Table I. 3 per cent. monohydric disodium citrate and 3 per cent. monohydric monosodium

investigation of the biochemical and physical changes occurring in the bloods stored in these solutions was undertaken in the hope of elucidating the mechanism of the beneficial effects. The changes studied were: the rates of potassium leakage from the red cells, of hydrolysis of ester phosphorus and of glucose breakdown, and the alterations in pH which took place when blood was kept in each of these diluents; the rate of spontaneous hæmolysis and the changes in osmotic fragility were also estimated; at the same time the amount of hæmolysis occurring on mixing the stored cells with fresh serum was determined as it was suspected that some of the more fragile cells might be hæmolysed on being brought into contact with the recipient's plasma after transfusion; the stored blood was also examined for inactive derivatives of hæmoglobin.

Studies of the biochemical changes taking place in blood stored in the standard M.R.C. diluent and in the Rous-Turner diluent were included for comparison.

METHODS.

In Vivo.

Estimation of Cell Survival.—Blood was taken from supposedly healthy donors into standard bottles containing the various solutions and stored at 3–7° C., until required for transfusion. On the day of transfusion, two bottles of identical age were taken, the supernatant plasma siphoned off and one bottle of concentrated red cell suspension prepared from the residual red cells. Approximately 500 c.c. of this suspension were transfused to various recipients, the time of transfusion varying from 40 to 120 minutes (with 6 exceptions). The recipients, with the exception of one case of Addisonian anæmia, were suffering from idiopathic or post-hæmorrhagic hypochromic anæmia. In all cases venous samples were taken immediately before and after transfusion, and further samples were taken at 24 hours, at 7 days, and thereafter at convenient intervals, usually including the 21st and 60th days after transfusion. In the majority of instances, survival was followed to completion, although only the figures for survival up to the end of 60 days are included in the tables of results.

The survival of the donor cells was estimated by a modification [Dacie and Mollison, 1943] of Ashby's method [1919]. Patients of group A or B were transfused with blood of group O. After transfusion the group O cells were counted after agglutinating the cells of the recipient. Allowance was made for the small number of un-agglutinated recipient's cells by estimating their number before transfusion.

Recording of Transfusion Reactions.—All cases transfused, whether with concentrated red cells or with whole blood, were personally

under the above conditions. Caramel would thus be less likely to be formed. The presence of oxygen also favours caramelisation and by autoclaving the bottles with the caps loose less colour is produced.

From the results quoted in Table I, it is clear that, of the solutions nos. 2–7, those that completely prevent caramelisation, namely 5 and 7,

TABLE I.

Diluent No.	Vol., c.cs.	Glucose, g.	Trisod. Cit., g.	Ac. Cit., g.	After autoclaving.		
					pH.	Colour arbitrary standard.	Per cent. glucose lost.
1	110	3	3·0	0	5·86	24 +	13·5
2	110	3	2·7	0·3	5·66	4 +	3·4
3	110	3	2·55	0·45	5·42	2 +	3·4
4	110	3	2·25	0·75	4·89	1 +	2·5
5	110	3	1·95	1·05	4·59	..	1·7
6	110	3	3·0 (Disod. Cit.)		4·92	1 +	1·7
7	110	3	3·0 (Monosod. Cit.)		3·68	..	1·0
8	120	6	0·64	0·36	4·64	..	0

are not so effective in delaying hæmolysis as some of the less acid solutions. At the same time it will be noted that some of the latter (nos. 2, 3, 4, and 6) are even more effective in delaying hæmolysis than is the M.R.C. solution. For this reason, and because with some of these solutions only very little caramel is formed, it was considered worth while to investigate their properties more fully, together with those of solution no. 8, which both prevents caramelisation and also inhibits hæmolysis satisfactorily.

The Main Investigation of the Solutions Nos. 2, 3, 4, 6, and 8.

The chief criterion adopted was the survival *in vivo* of transfused red cells stored in the various solutions. To determine whether any toxic substances had been formed during the storage, the reactions of the recipients to the transfusions were carefully recorded. Estimations of the recipients' alkali reserve before and after transfusion were made in a number of cases. In addition, when it became clear that the red cells of blood stored in these acidified solutions survived far better *in vivo* than those stored in the present alkaline M.R.C. diluent, an

In Tables VI and VII the results for solutions 2 and 8 and the M.R.C. diluent represent the actual figures found for potassium in the plasma diluent and an average of the figures found for inorganic phosphorus. In order that a direct comparison can be made the figures in the case of the Rous-Turner solution have been corrected to allow for the far greater volume of this diluent. The correction factor applied was obtained from the hæmatocrit estimations made upon blood stored in the Rous-Turner and M.R.C. diluents respectively. The inorganic phosphorus content of the cells was calculated from the plasma and whole blood figures and from the hæmatocrit reading.

Glucose.—The glucose contents of the red cells and plasma diluent mixtures were estimated on 0·2 c.c. samples by Hanes' [1929] method, the proteins being first removed as in Hagedorn and Jensen's [1923] method. Packed cells were obtained by centrifuging the blood at 3000 r.p.m. for one hour and removing all the supernatant plasma diluent and some of the top layer of cells every 10 minutes.

Hæmolysis of Stored Cells in Fresh Serum.—To 4 c.c. of one-day old serum, heparinised and warmed to 37° C., was added 1 c.c. of the cool stored blood. The mixture was kept at 37° C. for 30 minutes and then centrifuged. The hæmoglobin in the supernatant fluid was estimated by the same method as for the determination of spontaneous hæmolysis. In expressing the results allowance was made for the small amount of free hæmoglobin already present in the plasma of the stored blood. The proportions of blood and serum were chosen to imitate crudely the conditions of an average transfusion of 1000 c.c. citrated blood.

<div align="center">

RESULTS.

In Vivo.

</div>

The survival *in vivo* was estimated in 35 cases. The percentages of donor cells surviving at intervals after transfusion have been set out in Table IIA. As seen in this table, in the majority of cases blood stored in either solution no. 2 or no. 8 was used. The concentration of donor cells found immediately after transfusion has, as in a previous paper, been taken as a 100 per cent., so that the 24-hour figures may show an apparent increase due to a shift of fluid out of the blood-stream after transfusion. In cases in which survival was not estimated on exactly the day represented in the table, the appropriate figure was read off a graph after plotting from the observations available the percentage survival against the time after transfusion. In Table IIB the results are summarised and some figures for the survival of blood stored in the M.R.C. and Rous-Turner solutions have been included for comparison. These latter figures have been taken from the results of a previous series of trials [Mollison and Young, 1942].

The survival of blood stored in solution no. 2 for 14 days is very

observed throughout the transfusion. Temperature and pulse readings were made at half-hourly intervals for the duration of and for two hours after the transfusion.

Alkali Reserve.—This was estimated by the Conway method on whole blood samples collected under paraffin. In these cases whole blood rather than a concentrated red-cell suspension was used for transfusion. We are grateful to Dr. C. H. Gray for undertaking this part of the investigation.

In Vitro.

The behaviour of blood from at least 3–4 donors was investigated in the case of each solution tested. To prevent the bottles becoming infected through frequent opening, the contents of each were partitioned with sterile precautions into a number of 1-oz. bottles immediately after the blood had been collected. An undisturbed bottle was therefore available for every test.

Spontaneous Hæmolysis.—This was determined in the supernatant plasma diluent mixture from well-mixed and well-spun samples by matching the colour against dilute hæmoglobin standards. The results were expressed as a percentage of the total hæmoglobin content of any given sample.

Osmotic Fragility.—Median corpuscular fragility was estimated by the method of Dacie and Vaughan [1938]. All the precautions taken in the estimation of both spontaneous hæmolysis and osmotic fragility may be found in a previous paper [Mollison and Young, 1942].

Mean Corpuscular Volume.—This was determined from red-cell counts and hæmatocrit estimations.

pH Changes.—These were determined on whole blood diluent mixtures at room temperature by means of a glass electrode. Dr. M. Heppenstall kindly undertook this part of the investigation for us.

Formation of Inert Hæmoglobin Derivatives.—Mr. R. J. Bromfield examined for us the plasmas and laked red-blood corpuscles from blood stored for varying periods in diluents 2, 3, 4, 5, 6, and 8. A direct spectroscopic examination was made and in the case of plasma Schumm's test was also performed.

Estimations of methæmoglobin were also carried out on blood stored in diluents 2 and 8 and in the M.R.C. solution by Ammundsen's [1941] modification of the method due to van Slyke and Hiller.

Potassium.—Estimations were made on aliquots from trichloracetic acid filtrates of plasma by the method of Kramer and Tisdall [1921]. Three experiments were carried out and in each case blood from the same donor was taken into each of the solutions under investigation.

Inorganic Phosphorus.—Determinations were made by Martland and Robison's [1926] modification of Brigg's method on aliquots from trichloracetic acid filtrates of plasma and whole blood.

TABLE IIв.—SURVIVAL *IN VIVO* OF TRANSFUSED ERYTHROCYTES.   COMPARISON OF RESULTS OF PRESENT INVESTIGATION WITH THOSE OF PREVIOUS INVESTIGATION OF M.R.C. STANDARD SOLUTION AND ROUS-TURNER SOLUTION.

Preservative.	No. of cases.	Time of storage (days.)	Percentage survival after transfusion.			
			At 24 hours.	At 7 days.	At 21 days.	At 60 days.
M.R.C. . .	15	0–4	104	101	86	49
M.R.C. . . Solutions 2, 3, 4, 6, and 8	4 22 18 *	13 (av.) 14 (av.) 14 (av.)	88 100 100	77 93 93	60 78 82	27 38 45
M.R.C. . . Solutions 2, 3, and 6.	4 4	20 (av.) 20 (av.)	80 90	65 79	46 62	10 31
M.R.C. . . Solutions 2 and 8 Rous-Turner .	1 8 4	29 28 (av.) 26 (av.)	40 92 83	32 82 78	16 64 50	0 33 12

* See Table IIA.   Four atypical cases excluded.

similar to that of blood stored in solution no. 8, and the survival of both is markedly superior to that of blood stored in the M.R.C. solution for a similar period. Moreover, the survival of blood stored for approximately 28 days in solutions nos. 2 and 8 appears to be slightly better even than that previously found for blood stored in the Rous-Turner diluent. Survival in a few of the cases was atypical and these will be discussed below.

Solutions nos. 3, 4, and 6 were less fully investigated. The results were similar, though the impression is gained that the survival of the red cells after a given period of storage is slightly less good than with the other two solutions.

Reactions.—Of the 10 cases given 500 c.c. to 1040 c.c. of whole blood 4 had reactions. Of 36 cases given 300 c.c. to 530 c.c. of concentrated red-cell suspension 6 had reactions, see Table III. All these reactions were mild.

Alkali Reserve.—In 5 cases the alkali reserve was estimated before and immediately after a rapid transfusion of 600–950 c.c. of blood stored for 12 to 13 days in solutions nos. 2 and 8. In 4 of the 5 cases a very small increase of alkali reserve was noted after transfusion, see Table IV.

In Vitro.

Spontaneous Hæmolysis.—The results presented in Table V show clearly that the rate of hæmolysis in diluents 2, 3, 4, 6, and 8 was

TABLE IIA.—INDIVIDUAL RESULTS. SURVIVAL *IN VIVO* OF TRANSFUSED ERYTHROCYTES.

Preservative.	No. of cases.	Time of storage (days).	Percentage survival after transfusion.			
			At 24 hours.	At 7 days.	At 21 days.	At 60 days.
Solution 2 .		14	101	93	85	49
		14	105	92	86	56
		14	109	101	85	53
		14	101	92	86	51
		14	107	91	78	46
		14	95	97	84	?
	*	14	100	91	61	19
	7 (av.)	14	103	94	81	46
Solution 8 .		14	116	103	97	44
		14	91	92	79	40
		14	103	90	96	59
		14	100	94	81	52
		14	99	95	87	42
		14	98	92	80	28
	*	14	96	84	55	0
	7 (av.)	14	100	93	82	38
Solution 3 .		12	94	77	68	45
		15	97	93	78	33
Solution 6 .		14	106	100	60	33
		14	81	105	82	48
		14	95	88	74	38
	*	14	107	98	56	5
Solution 4 .		14	98	86	91	53
	*	16	98	91	63	0
Solutions 2, 3, 4, 6, and 8.	22 (av.)	14	100	93	78	38
Do. do. (Cases * omitted)	18 (av.)	14	100	93	82	45
Solution 2 .		22	92	67	45	26
		27	77	72	52	20
		28	85	84	70	43
		30	85	79	61	30
		47	50	35	35	12
Solution 8 .		27	111	94	82	56
		28	92	84	65	32
		29	106	95	78	49
		29	99	88	69	17
		30	70	58	37	16
Solution 3 .		21	93	91	73	42
Solution 6 .		19	87	82	65	24
		19	88	75	65	34

* 4 cases in which elimination was unusually rapid.

was only gradual and the rise from the initial values was found to be approximately equal for each diluent.

Cells stored in Murray's diluent no. 8 rapidly became very fragile to hypotonic solutions; the fragility increased rather more rapidly during storage than in the other acid solutions.

Mean Corpuscular Volume.—It was found that the mean corpuscular volume was increased by every solution immediately the blood was
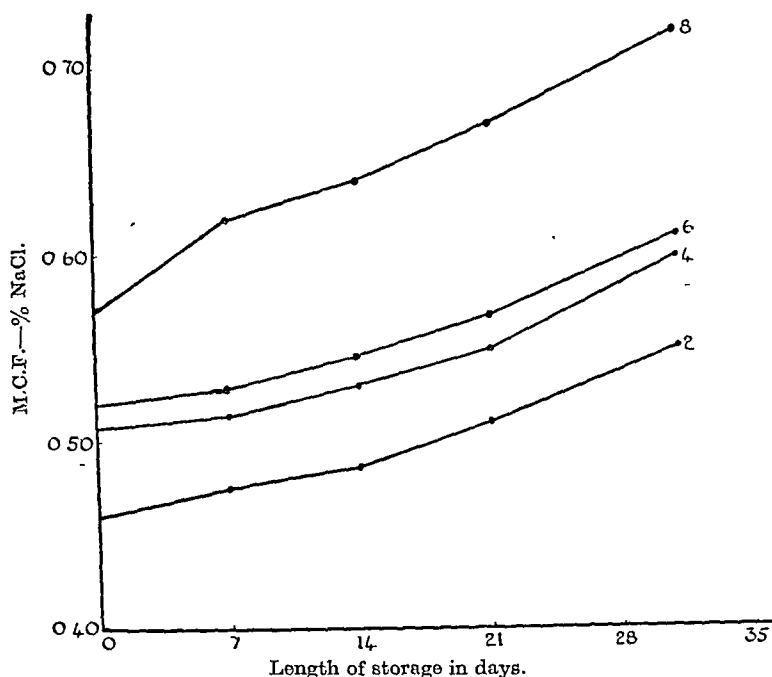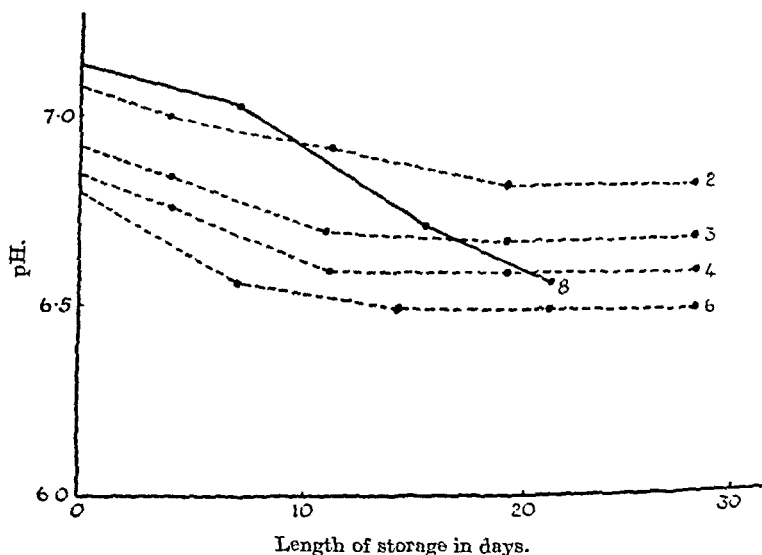

FIG. 1.—Osmotic fragility changes in stored blood.
(The figures at the side of the graphs indicate the number of the diluent tested—see Table I.)

taken into it. This change was more pronounced in the more acid solutions. There was very little change at the end of 4 weeks' storage.

pH Changes.—The results are represented in fig. 2. In the case of solutions nos. 2, 3, 4, and 6 it will be seen that the initial reaction of the blood diluent mixtures ranged from pH 7·1 to pH 6·8, varying with the pH of the diluent, for the total quantity of electrolyte was roughly the same in each. The pH then fell slowly during 14 days' storage to pH 6·85 to 6·5 when equilibrium seemed to be established in every case. With diluent no. 8, in which the total electrolyte content was less than in the other solutions, the fall throughout storage was greater, from pH 7·15 to pH 6·6 in 21 days.

Potassium.—The results of experiments to determine the effects of diluents nos. 2 and 8 and the M.R.C. and Rous-Turner diluents upon the

definitely slower than in the M.R.C. solution. All these solutions were about equally effective in decreasing the rate of hæmolysis during storage.

TABLE III.—TRANSFUSION REACTIONS.

Transfusion fluid.	No. of cases.	No reaction.	Reaction.			
			Pyrexia, > 100° F.	Rigor.	Symptom-less urticaria.	Vomiting muscle pain.
Whole blood .	10	6	2	0	2 *	0
Conc. red cell susp.	36	30	4	2	1	2

* One case complained of itching.

TABLE IV.—ALKALI RESERVES.

Diluent no.	Days of storage.	Whole blood given, c.c.	Time of transf. in mins.	Alkali reserve (vol. CO_2 per cent.).	
				Before transf.	After transf.
2	13	600	35	49	55
2	12	950	80	59	58
2	13	900	75	63	65
8	13	700	90	62	68
8	12	950	50	56	58

TABLE V.—SPONTANEOUS HÆMOLYSIS.

Diluent.	Per cent. hæmolysis. Time of storage in days.					
	0.	7.	14.	21.	28.	35.
M.R.C. . . .	..	0·1	0·3	0·6	1·5	4·4
Rous-Turner . .	..	0	0	0·2	0·3	0·6
Diluent no. 2 . .	..	0	0·2	0·3	0·65	1·60
,, ,, 4 . .	..	0	0·1	0·3	0·5	
,, ,, 6 . .	..	0	0·2	0·3	0·5	
,, ,, 8 . .	..	0	0·2	0·3	0·5	1·4

Osmotic Fragility.—When blood was taken into the acid solutions 2, 4, and 6, the median corpuscular fragility was immediately increased, see fig. 1. The more acid the solution the greater was the observed increase. The progressive increase in osmotic fragility during storage

7·35, lost potassium rapidly and at approximately the same rate as erythrocytes preserved in the M.R.C. solution.

Inorganic Phosphorus.—This increased progressively in the erythrocytes of blood stored in the M.R.C. solution and a small rise was also

TABLE VII.—CHANGES IN INORGANIC PHOSPHORUS DURING STORAGE—
EXPRESSED AS MG. PER CENT.

(Average figures for three experiments.)

Diluent.	Cells.				Plasma-Diluent.			
	Time of storage in days.				Time of storage in days.			
	0.	7.	14.	21.	0.	7.	14.	21.
M.R.C. . . .	3·3	6·3	19·4	25·1	1·5	2·3	2·6	4·0
Rous-Turner . .	6·0	11·5	16·5	19·7	2·0	2·9	3·8	4·9
Diluent no. 2 . .	8·2	13·0	26·5	30·0	1·5	2·8	3·2	4·4
,, ,, 4 . .	6·5	11·0	24·5	33·0	1·5	4·0	5·5	7·0
,, ,, 6 . .	8·5	10·5	27·0	34·0	1·5	4·5	5·2	6·5
,, ,. 8 . .	7·5	11·0	20·0	27·0	1·5	4·1	4·7	6·2

TABLE VIII.—CHANGES IN TOTAL GLUCOSE CONTENT OF STORED BLOOD—
RESULTS EXPRESSED AS G. PER CENT.

	M.R.C.		No. 2.		No. 8.		Rous-Turner.	
	0 days.	28 days.	0 days.	28 days.	0 days.	28 days.	0 days.	28 days.
1. {Cells . . .	0·56	0·42	0·55	0·41	0·92	0·79	2·28	2·12
{Plasma-diluent .	0·74	0·66	0·70	0·59	1·36	1·25	2·85	2·74
2. {Cells . . .	0·54	0·40	0·54	0·39	0·90	0·78	2·06	1·90
{Plasma-diluent .	0·70	0·61	0·71	0·65	1·31	1·23	2·63	2·45
3. {Cells . . .	0·55	0·42	0·53	0·42	0·95	0·81	2·37	2·23
{Plasma-diluent .	0·72	0·63	0·69	0·60	1·39	1·30	2·97	2·84

found in the plasma, see Table VII. Increasing the acidity of the blood diluent mixture increased the rate of hydrolysis of cell ester phosphorus and increased the rate of appearance of inorganic phosphorus in the plasma. In the Rous-Turner diluent the changes took place rapidly during the first fortnight's storage and thereafter more slowly.

Glucose.—From the results summarised in Table VIII it will be seen that glycolysis took place in both the cells and plasma of blood

extent of permeability to cations, as represented by potassium leakage from the cells, are tabulated in Table VI. It would appear that decrease of the initial pH of a blood diluent mixture from 7·45 to 7·15 retards

Length of storage in days.

FIG. 2.—pH changes in stored blood.

(The figures at the side of the graphs indicate the number of the diluent tested—see Table I.)

TABLE VI.—CHANGES IN PLASMA POTASSIUM—EXPRESSED AS MG. PER CENT. IN PLASMA-DILUENT.

(Actual figures in three experiments.)

Days of storage.	Diluent.											
	Present M.R.C.			Rous-Turner.			Diluent no. 2.			Diluent no. 8.		
	(1).	(2).	(3).	(1).	(2).	(3).	(1).	(2).	(3).	(1).	(2).	(3).
0	14·1	13·1	12·1	18·1	21·0	17·3	15·2	12·6	13·8	16·0	12·2	14·1
7	98	96	105	96	105	101	76	80	86	65	80	80
14	121	118	136	131	138	121	96	105	99	87	105	90
21	146	136	142	149	142	138	121	118	127	102	126	121
28	150	145	150	157	150	156	141	135	136	126	132	144

the leakage of potassium, for not only does the plasma potassium rise more slowly but the value attained at 28 days is lower. Cells stored in Rous-Turner solution, the initial pH of the blood diluent mixture being

transfused to them [Wiener and Peters, 1940]. Further support was provided by the examination of the record of a case reported by Dacie and Mollison [1943]. In this, diminished survival was found in one subject out of a group of 6, all of whom were suffering from the same variety of anæmia. This one subject proved to be Rh negative and it was shown that both donors were Rh positive. The donor blood survived well initially, but was then eliminated far more rapidly than in the other 5 cases, destruction being complete by the 60th day. Examination of this recipient's serum shortly afterwards revealed the presence of weak anti-Rh agglutinins. This case presents certain similarities to the anomalous cases in the present series. However, when the other cases in the present series were investigated, it was found that 2 out of 3 of them were Rh positive (the third subject was not available for testing). No definite opinion can therefore be given as to whether the unusually rapid elimination of these four cases was caused by some abnormal hæmolytic mechanism in the recipients or by the production of specific immune antibodies.

It has been thought that blood stored in acid or caramelised preservatives might be "toxic." However, the reactions encountered in this series of transfusions were not qualitatively or quantitatively different from those seen by us when using blood stored in the M.R.C. diluent. One case received without reaction in 90 minutes 800 c.c. of whole blood stored for 7 days in solution no. 5 (probable pH 6·45).

In the 5 cases receiving whole blood at a relatively rapid rate, there was no lowering of the alkali reserve. Wurmser *et al.* [1942] report similar findings. It is interesting to note that de Souza and Hocking [1935] showed that intramuscular injection of both sodium citrate and citric acid produced a marked rise in the alkali reserve in the cat.

So far as is known, no evidence has been produced to show that the intravenous injection of caramel is in any way dangerous to man. In guinea-pigs Hanzlik and Karsner [1924] found ho reaction after the intravenous injection of large quantities (3 c.c. of a 0·5 per cent. solution), though at autopsy distension and congestion of the lungs with hæmorrhages were found. It should be noted that the authors found the same changes after the intravenous injection of 1·2–2 c.c. of 2–5 per cent. sodium citrate solution. Moreover, the animals given citrate solution reacted with marked symptoms and two died promptly. Under the conditions of their experiment, therefore, it would appear that caramel was less toxic than sodium citrate.

Formation of Inert Hæmoglobin Derivatives during Storage.

Brooks [1931] found that at 25° C., in the presence of oxygen, hæmoglobin solutions were readily converted into methæmoglobin at pH 7·04 and pH 6·67, but that the temperature coefficient was high;

stored in the four solutions studied and, further, that glucose was broken down in the cells more rapidly than in the plasma. The changes taking place were similar in diluents nos. 2 and 8 to those occurring in the M.R.C. solution.

Formation of Hæmoglobin Derivatives.—Direct spectroscopic examination failed to reveal the bands either of methæmoglobin in the laked red cells or of methæmalbumin in the plasma of any of the bloods examined at the end of 5 weeks' storage. Plasma from blood stored in solutions nos. 6 and 8 for 1 month, however, gave a positive Schumm's test.

Blood stored in diluents nos. 2 and 8 was found after 30 to 39 days to contain 0·8 to 3·4 per cent. of methæmoglobin. Similar quantities were also found in blood stored in the M.R.C. solution.

Hæmolysis of Stored Cells in Fresh Serum.—With blood stored for 4 weeks or less, no detectable hæmolysis occurred under the conditions of the experiment.

<center>DISCUSSION.</center>

From the results of the survival tests *in vivo* it is apparent that these citric acid-sodium citrate-glucose mixtures are far better preservatives than the alkaline M.R.C. sodium citrate-glucose mixture. This superiority is quite distinct when the period of storage is 14 days and is even more pronounced when the period of storage is 28 days.

In a previous paper [Mollison and Young, 1942] it was mentioned that with a given preservative and a given length of storage there appeared to be little variation in survival between one case and the next, provided that the recipient was suffering from a simple secondary anæmia. In the present series of cases, however, although, as will be seen from Table IIA, survival is consistent in the majority of cases within the various groups, there are 4 cases in which survival was distinctly shorter. In these cases survival was not significantly below the average for cases in the group for the first 7–21 days after transfusion, but thereafter there was a rapid falling off so that elimination was virtually complete at the end of 40–60 days. This type of elimination is in distinct contrast with that usually found after the transfusion of old stored blood, in which initial destruction is rapid, but in which some of the transfused cells may survive for long periods [Mollison and Young, 1940].

Because of the initially good survival and later rapid destruction in these four anomalous cases, it was considered that the slow production of immune antibodies to some blood-group factor contained in the donor's erythrocytes might be responsible. This supposition appeared to be supported when the first of the four subjects to be investigated was found to be Rh negative, since it is now known that such subjects are liable to form immune antibodies when Rh positive blood is

transfused to them [Wiener and Peters, 1940].   Further support was provided by the examination of the record of a case reported by Dacie and Mollison [1943].   In this, diminished survival was found in one subject out of a group of 6, all of whom were suffering from the same variety of anæmia.   This one subject proved to be Rh negative and it was shown that both donors were Rh positive.   The donor blood survived well initially, but was then eliminated far more rapidly than in the other 5 cases, destruction being complete by the 60th day.   Examination of this recipient's serum shortly afterwards revealed the presence of weak anti-Rh agglutinins.   This case presents certain similarities to the anomalous cases in the present series.   However, when the other cases in the present series were investigated, it was found that 2 out of 3 of them were Rh positive (the third subject was not available for testing).   No definite opinion can therefore be given as to whether the unusually rapid elimination of these four cases was caused by some abnormal hæmolytic mechanism in the recipients or by the production of specific immune antibodies.

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Brooks [1931] found that at 25° C., in the presence of oxygen, hæmoglobin solutions were readily converted into methæmoglobin at pH 7·04 and pH 6·67, but that the temperature coefficient was high;

therefore it seemed unlikely that much would be formed at 4° C. in the presence of the reducing substance glucose. As has been stated, no methæmoglobin could be demonstrated spectroscopically; Harrison [1937] quotes Bloem's statement that 0·3 per cent. methæmoglobin can be detected in the presence of large quantities of hæmoglobin, though Ammundsen [1941] considers that 15–20 per cent. must be present before it can be identified spectroscopically. The 0·8–3·4 per cent. methæmoglobin found by the method of Van Slyke and Hiller in blood stored in diluents nos. 2 and 8 and in the M.R.C. solution is very small and, moreover, such values were often found by Ammundsen [1941] in the blood of normal subjects.

Changes in the Properties In Vitro *and their Relation to Survival* In Vivo.

The results confirm the findings of Cotter and McNeal [1938], Maizels and Whittaker [1940], and Wurmser *et al.* [1942], that hæmolysis is inhibited by increasing the hydrogen ion concentration of a blood diluent mixture. They also agree with the results of Maizels [1935], Jeanneney and Servantie [1939], and Scudder and Smith [1940], that potassium leakage is inhibited by a certain increase in hydrogen ion concentration. Even more striking is the demonstration that the survival *in vivo* of blood stored in acid solution is equal or superior to the survival of blood stored in the Rous-Turner mixture which had previously been shown to be superior to the current M.R.C. solution. The rates of hæmolysis in acid solutions are similar to the rate of hæmolysis in the Rous-Turner mixture, all showing less than 1 per cent. hæmolysis after 28 days' storage, against the 1·7–2 per cent. exhibited by the M.R.C. solution. In a previous paper [Mollison and Young, 1942] it was shown that inhibition of hæmolysis may be a fallacious guide to the state of preservation of biological activity of the erythrocytes as judged by their subsequent survival *in vivo*. The test, however, could have a negative value, for solutions in which rapid hæmolysis took place had always to be rejected. This conclusion agreed with an earlier warning of Maizels and Whittaker [1940] that a solution which delayed hæmolysis *in vitro* might prove to have little effect in prolonging survival *in vivo*. In view of the rates of hæmolysis compared above, it is suggested that a more specific statement could be made to the effect that if more than 1 per cent. hæmolysis is present after 21 days' storage, then the solution under trial is not likely to be better than the M.R.C. diluent.

Changes in the osmotic fragility of stored red cells were shown previously to be a very fallacious guide to their state of preservation. The additional data from the acid solutions emphasise again that there is no constant relationship between osmotic fragility and subsequent cell survival *in vivo*.

In the body, a difference in the relative proportion of the bases sodium and potassium exists between the cells and the plasma. Potassium is the predominant base in the erythrocytes and little or no sodium is thought to be present; sodium, on the other hand, predominates in the plasma and the concentration of potassium is only about 1/20th of that found in the erythrocytes. *In vitro*, the erythrocyte membrane becomes permeable to cations and thus the red cells gain sodium and lose potassium, while at the same time the plasma loses sodium and gains potassium. One might suppose that if the *in vivo* relationship could be maintained or its disturbance limited during storage, the erythrocytes would survive in a recipient for longer periods. The experiments to test this hypothesis were disappointing, for, although in the acid solutions tested the leakage of potassium was definitely decreased, erythrocytes stored in the Rous-Turner diluent lost potassium at the same rate as in the M.R.C. solution. Scudder *et al.* [1939] also found that leakage of potassium was rapid from erythrocytes kept in the Rous-Turner solution.

It would appear, therefore, that the relationship of the cations in the body is not related to the maintenance of the integrity of the red-cell membrane, but that the red cell acts as a vehicle for potassium in some general metabolic function. The results of Maizels and Patterson [1940] would indicate this, for they showed that stored red cells which have acquired sodium lose it against a high concentration gradient when they are transfused to a recipient and yet continue to survive well in the recipient's circulation.

In the body, a balance also exists between the breakdown of ester phosphorus to inorganic phosphate and its resynthesis. Outside the body, the same processes persist for a time. Guest [1932] in short-term experiments showed that the inorganic phosphate content of the cells does not rise until the blood glucose is completely broken down. Martland [1925] found that synthesis of ester phosphorus takes place above pH 7·35 and hydrolysis below pH 7·29; glycolysis accompanied the synthesis above pH 7·35, while the changes occurring in the glucose when the pH was below 7·3 were insignificant. With longer periods of storage Aylward, Mainwaring, and Wilkinson [1940] demonstrated that the addition of glucose to blood stored in sodium citrate significantly decreased the rate of hydrolysis of ester phosphorus within the red cells. Maizels [1941 *b*] confirmed this and suggested that the resynthesis of ester phosphorus was permitted by the added glucose; in its absence the small amount of glucose normally present was rapidly broken down and thus resynthesis of ester phosphorus could not occur.

It was therefore of great interest to test whether this beneficial effect of added glucose on the hydrolysis of ester phosphorus would be present with the Rous-Turner and acid diluents. Wurmser *et al.* [1942] had already shown that breakdown of blood sugar was reduced in acid

solutions; Martland's results suggested that the hydrolysis of phosphoric ester would be increased in acid solutions, but it was possible that the presence of added glucose would counteract this. In point of fact, as has been noted, the breakdown of glucose in the red cells of blood stored in diluents nos. 2 and 8 was practically the same as that found with the M.R.C. solution, but inorganic phosphate was released in the acid diluents at a rate comparable with that taking place in blood stored without glucose. Glycolysis was most rapid in the Rous-Turner diluent, while the rate of ester phosphorus breakdown was comparable with that taking place in the acid diluents for the first fortnight and then decreased. The maintenance of a normal balance of hydrolysis and synthesis of ester phosphorus does not thus seem necessarily to be a property of a good blood preservative.

Practical Application of Results.—The results reported above form good ground for the recommendation of certain citric acid-sodium citrate-glucose mixtures as blood preservatives. Their advantages over previously described solutions may be summarised as follows:—

1. The entire solution can be autoclaved without producing gross discoloration.

2. The preservative effect as judged by survival tests *in vivo* is considerably better than that of the standard M.R.C. trisodium citrate-glucose solution. Thus, survival after 14 days' storage is practically as good as that of fresh blood instead of showing a definite falling off (as is the case with blood stored in the M.R.C. solution). Even after 28 days' storage, the erythrocytes of blood stored in these acid solutions are not rapidly destroyed, in fact approximately 60 per cent. survival is found at the end of 21 days after transfusion. This survival rate is admittedly but little better than that of blood stored in the Rous-Turner solution. However, the latter is so bulky that it has generally been regarded as impracticable, particularly when the plasma of stored blood is to be salvaged.

It may be pointed out that none of the solutions tested in this series combines the merits of all. For instance, solutions nos. 2, 3, 4, and 6 become definitely discoloured on autoclaving. Solution no. 8 does not become discoloured on autoclaving but contains so little citrate that clotting is liable to occur unless meticulous care is taken during the collection of the blood. Nevertheless, many possible combinations of sodium citrate, citric acid and glucose remain, and tests are proceeding.

SUMMARY.

1. Certain citric acid-sodium citrate-glucose mixtures have been tested and found to be satisfactory for use as blood preservatives. The use of these solutions is recommended for the following reasons:—

(a) Red cells stored in these solutions survive better in the recipient's circulation after transfusion than when stored in any other solution yet tested.

(b) The whole mixtures can be autoclaved with the production of little or no caramel.

(c) The transfusion of blood stored in these solutions has not been accompanied by the production of any untoward results.

(d) The amount of methæmoglobin formed when blood is stored with these solutions is not significantly greater than the amount formed when the usual trisodium citrate-glucose solution is used.

2. The rates of release of inorganic phosphorus, of potassium shift, of glycolysis, of hæmolysis and of alteration in the osmotic fragility of blood stored in these solutions and in the Rous-Turner and standard M.R.C. citrate-glucose solutions were investigated. The changes observed were not found to be correlated with the ability of the erythrocytes to survive *in vivo* after transfusion, except that *in this series* delay in hæmolysis was associated with improved survival *in vivo*.

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THE HISTOLOGY OF THE ISOLATED PERFUSED LUNG. By

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THIS paper is an account of the histological changes found in dogs' lungs which had been isolated from the body and perfused with heparinised blood under negative pressure ventilation for a period of 3½ to 7 hours. The perfusion technique has been fully described by Daly, Hebb, and Petrovskaia [1941]. The lungs were examined in 20 perfusion experiments, in most cases both the right and left lungs were taken, making a total of 34 lungs examined in all. The lungs of 5 normal dogs removed under chloralose or nembutal anæsthesia served as controls.

HISTOLOGICAL METHODS.

From each lung three samples, each about 4 cm.³, were cut out and fixed in 8 per cent. neutral formol saline. The samples were taken from different parts of the lung in different experiments and collectively, in the whole series, they covered practically all parts of the lung from the root to the pleura. No attempt was made to assess the relative distribution of the changes in the different parts of the lung, but the survey was sufficiently wide to give a general picture of the perfused lung and to include any changes which might have been localised to particular regions of the lung. Formol saline gave satisfactory fixation. Other fixatives—Bouin, Susa, Zenker, Carnoy, mercuric chloride-formol—gave no better, and in most cases inferior, results.

After fixation the pieces were trimmed down to about 1 cm.³. Paraffin sections were made, using dioxane and vacuum embedding. In some cases frozen and celloidin sections were also made.

With regard to the staining of paraffin sections, ordinary H and E proved rather inadequate especially in respect of the alveolar walls, the walls of small blood vessels and the recognition of extravascular red cells. Mallory's, Heidenhain's Azan and Masson's Trichrome methods were all too coarse. The following three methods all gave good results: (1) Hæmalum and Biebrich Scarlet, (2) Celestin Blue and van Gieson, (3) Hæmalum, Aurantia, and Aniline Blue. This last method has been described elsewhere [Marshall and Trowell, 1943],

and can be especially recommended as a general purpose stain for the lung; it was in fact originally devised for this purpose. Frozen and celloidin sections do not permit of very precise selective staining, and a general stain, such as Hollande's Chlorocarmine, gave the best results.

Some Histological Features of the Normal Dog's Lung.

The normal control lungs showed a few features which seem worthy of mention, if only to point out that they are not the result of perfusion.

1. The bronchial walls contain rather more connective tissue than is the case in other species (rat, mouse, guinea-pig, rabbit, monkey, man). The bronchial glands mostly have very large lumina with flattened secreting cells. The bronchial epithelial cells often contain quite large fat droplets.

2. Compared with other species, the alveolar ducts (of Miller) are very prominent in sections, owing to their greater length and well-developed muscular wall.

3. The alveolar macrophages generally contain a good deal of carbon and are therefore readily recognised. In nearly all the lungs examined (both normal and perfused) small areas were found in which the alveolar macrophages were greatly enlarged, the cytoplasm being filled with small lipoid droplets staining with Sudan IV. Many of these cells were binucleate; the nuclei were usually very pale staining, but sometimes pyknotic. These cells were mainly restricted to small foci scattered throughout the lung and were accompanied by no other pathological change. They occurred in the alveolar wall, but more commonly as rounded cells lying free in the alveoli; often several adjacent alveoli were entirely filled with them. These lipoid-filled macrophages found in the lungs of apparently normal dogs seem to correspond to the "foam cells" of human pathology, where they are associated with chronic inflammation, and they probably have the same significance here.

4. In paraffin sections the arteries are always surrounded by an artifact shrinkage space and the outer part of the adventitia is usually somewhat torn up. This shrinkage space was not found in celloidin sections and so it appears to be due to paraffin. A similar but less extensive periarterial space was often found in frozen sections, but here it was due to tearing of the loose perivascular connective tissue during the cutting or subsequent handling of the sections and could be avoided by taking suitable precautions. It seems that this periarterial zone is a region of mechanical weakness in the lung, the perivascular connective tissue and the outer layers of the adventitia being very readily torn. This point will be referred to again later in connection with the perfused lung. There was no homologous shrinkage space round the bronchi or veins.

Histological Changes in the Perfused Dog's Lung.

A. *Constant Changes* (*i.e.* present in some part or other of every lung).—These changes were all confirmed in frozen and celloidin sections.

1. *Œdema,* manifest histologically by—

> (i) *Alveolar Exudate.*—In many parts of the lung the alveoli were wholly or partly filled with a homogeneous, lightly staining, basophilic exudate (figs. 1, 2). Occasionally the exudate was finely granular but it never contained fibrin or red cells. With aniline blue - orange G the exudate stained predominantly blue; according to Short [1941] this indicates a protein content of less than 1 per cent. Taken as a whole the histological evidence points to a low protein content of the exudate, comparable to that of œdema fluid elsewhere.
>
> (ii) *Distension of Periarterial Lymphatics.*—Around many of the arteries the perivascular lymphatics were considerably distended, the lymphatic vessels being filled with cell-free fluid similar in staining reactions to the alveolar exudate (figs. 1, 3). Rarely the perivenous lymphatics were similarly distended, but the peribronchial never. In fact the peribronchial lymphatics could rarely be recognised in any sections of the dog's lung, normal or perfused, though according to Miller [1937] they are even more numerous than the periarterial. The subpleural lymphatics were sometimes slightly distended with fluid. The periarterial lymphatics had a distinct and definite wall; they were not confused with the periarterial shrinkage spaces which occur in paraffin sections. They were most clearly seen in celloidin sections, for here there are no artifact shrinkage spaces.
>
> (iii) *Œdema of Arterial Walls.*—In a few arteries the collagen bundles of the adventitia and perivascular connective tissue were separated by interstitial collections of œdema fluid (fig. 2). This was not a very frequent finding and was not found in every lung.

In connection with the œdema it should be mentioned that the lungs never showed any congestion; the vessels, including the capillaries, were of normal size and filling. The lumina of the bronchi and bronchioles sometimes contained a little œdema fluid, but for the most part were quite free from exudate or debris.

2. *Periarterial and Peribronchial Hæmorrhage.*—Many of the pulmonary arteries were surrounded by a zone of red cells lying free in the periarterial connective tissue, which was disrupted. Around such arteries distended periarterial lymphatics could often be recognised, but the usual shrinkage space was not present (fig. 3). When the hæmorrhagic zone was narrow, the red cells were strictly periarterial in position. When the hæmorrhagic zone was wider, red cells were found infiltrating the adventitia of the artery and also extending round the adjacent bronchus, tracking round in the peribronchial connective tissue, often completely surrounding the bronchus (fig. 4), and in severe

cases infiltrating the whole thickness of the bronchial wall right in to the epithelium. The appearances indicated that the hæmorrhage was primarily periarterial in position and that, when extensive, it spread round the adjacent bronchus and also into the arterial and bronchial walls, the final picture in severe cases being a general hæmorrhagic infiltration of the whole broncho-arterial tract. This hæmorrhagic condition of the broncho-arterial tracts could be clearly seen on naked-eye examination of the cut surface of the fresh lung—it extended for considerable distances along the tracts. The periarterial hæmorrhage was most frequently met with, and was, relative to the size of the artery, most extensive in arteries of 0·2–0·5 mm. internal diameter; of such vessels, in any one lung, usually more than half the total number were affected. It also occurred in larger arteries, even in the main branches at the root of the lung. It was uncommon in arteries below 0·15 mm., and was never found in arteries below 0·1 mm. or in arterioles. The diameters given refer to measurements on paraffin sections.

3. *Collection of Polymorphonuclear Leucocytes in Small Blood Vessels.*—In many parts of the lung large numbers of polymorphs were present in the capillaries and venules; often the lumen was entirely filled with polymorphs to the exclusion of red cells. In the capillaries the polymorphs occurred singly and also in groups of two to six (fig. 6). On the whole the greater number seemed to be in the venules; here they often occurred in large masses, completely filling the vessel (fig. 5). Occasionally polymorphs were seen in an arteriole, but this was uncommon. In all cases the polymorphs were separate and discrete and of normal appearance, there was no evidence of clumping or disintegration. In one experiment in which the vessels were washed through with saline at the end of the perfusion, no polymorphs were found on subsequent histological examination of the lung. In two experiments in which the lung was perfused for only a few minutes, the heparinised blood being passed through only once, polymorphs were found in the usual numbers. These experiments indicate that the polymorphs are removed from the circulating blood at an early stage in the perfusion, that they remain in, and probably obstruct, some of the vessels throughout the perfusion, but they can readily be dislodged by saline perfusion.

The fact that polymorphs disappear from blood perfused through an isolated lung was discovered by Bickford and Winton [1924], but they did not follow up the fate of the polymorphs. In the perfusion experiments which provided the material for the present investigation, leucocyte counts were made at intervals on the circulating blood and the findings of Bickford and Winton were confirmed. The number of polymorphs rapidly decreased in the early stages of the perfusion and thereafter more slowly, but progressively, throughout the experiment. These findings will be reported in detail elsewhere; the point

of interest here is that the disappearance of the polymorphs from the circulating blood can be accounted for by their accumulation in large numbers in some of the small vessels of the lung, particularly the venules. Why the polymorphs should stick in the small vessels has not been discovered. Some evidence was obtained that under the artificial conditions of perfusion the polymorphs in the circulating blood clumped together, in a manner analogous to the agglutination of red cells; such clumped masses of polymorphs would doubtless act as emboli and stick in the small vessels of the lung. The difficulty of this interpretation, however, is that the larger masses of polymorphs were found in the venules, not the arterioles. An alternative explanation would be that primarily the polymorphs adhere to the walls of the vessels and later also to each other. If this were so the accumulation might be expected to start in the venules rather than the arterioles. Histologically, however, there was no evidence of any concentration of the polymorphs against the walls of the vessels (figs. 5, 6).

4. *Dilated Bronchi and Bronchioles.*—In the normal controls, as is usual, the bronchial epithelium was highly convoluted. In the perfused lungs these convolutions were largely smoothed out and the lumen correspondingly wider. The convoluted epithelium and small lumen of the bronchi and bronchioles seen in sections of normal lung is due to post-mortem contraction of the bronchial muscle and does not represent the condition *in vivo*. The comparatively dilated state of the bronchi and bronchioles in histological sections of the perfused lung therefore does not prove that the bronchial tree is actually more dilated in the perfused lung than in the normal animal, but it does suggest a loss of tone or reactivity on the part of the bronchial muscle. This is to be expected, because in these experiments the bronchial circulation was not perfused.

5. *Vascular Congestion of Bronchial Walls.*—In most of the perfusions the venous outflow was collected by means of a canula tied in the left auricle. In all such experiments the blood vessels in the bronchial wall, especially the capillaries and venules, were greatly dilated and filled with blood. In other experiments the pulmonary veins were slit widely open and the outflow collected by drainage; in such cases the bronchial vessels were collapsed and generally empty. It seems likely that in the former experiments the canula imposed some degree of obstruction causing a rise of pulmonary venous pressure and a back flow of blood into the bronchial circulation from the region of capillary anastomosis of the bronchial and pulmonary circulations.

6. *Alveolar Walls, Bronchial Epithelium, Vascular Endothelium* showed no change as compared with the controls.

B. *Occasional Findings.*—In two experiments a single pulmonary artery was found filled with and distended by an amorphous mass of non-cellular material, apparently protein in nature but histologically

not fibrin.   In one case the mass contained a good number of diphtheroid bacilli.   It was probably debris or dirt picked up from the perfusion apparatus.   In two other experiments an area of lung was found in which the capillaries were entirely empty of blood and the arteries and arterioles contained plasma only.   This was probably an area which escaped perfusion, possibly owing to arterial blockage similar to that described above.

The three main changes described—namely the œdema, the peri-arterial hæmorrhage, and the intravascular collection of polymorphs—were always patchy in distribution, none of them was ever present throughout the whole lung, and generally some areas could be found which were quite free from any of these changes.   As far as could be judged the distribution of these changes was quite random and they were unrelated to one another.   For instance, massive periarterial hæmorrhage was often found in parts of the lung which were quite free from œdema, and there was certainly no relation between the distribution of polymorphs and that of alveolar exudate.   The bronchial dilation and the bronchial congestion (when present) were always uniform throughout the lung.

DISCUSSION.

The histology of the perfused lung does not appear to have been previously described.   Histological changes in the lung of the heart-lung preparation have been described, and it is interesting to compare them with those found in the perfused lung.   Lambert and Gremels [1926] used the dog's heart-lung preparation with defibrinated blood. Their preparations only survived 4 hours or less, the terminal event in all cases being the rather sudden onset of acute œdema of the lungs (froth pouring from the trachea).   In the isolated perfused lung, using heparinised blood, œdema of this severity does not occur, even after 7 hours' perfusion; such œdema as does occur is relatively mild, patchy in distribution, and develops slowly during the course of the experiment.   Lambert and Gremels found the following histological changes in the lungs after the onset of the acute œdema: (1) damage to vascular endothelium as evidenced by absence, poor staining or fragmentation of the endothelial nuclei in arteries, capillaries, and veins; (2) swelling of the "cells in the walls of the arteries and veins" (presumably muscle fibres and fibroblasts); (3) swelling and desquamation of bronchial and alveolar epithelium; (4) the alveoli were filled with desquamated cells and amorphous masses—quite different from the coagulated fluid found in human pulmonary œdema; (5) the arterioles were sur-rounded by a cuff of red cells, which they attributed to diapedesis through the arteriole wall.   Strictly speaking none of these changes was met with in the isolated perfused lung; it is true that alveolar

exudate occurred, but it was a different sort of exudate, and that periarterial hæmorrhage occurred, but it was around the arteries only, never the arterioles.   Lambert and Gremels thought that the findings in the heart-lung preparation represented a damage to vascular endothelium by some toxic agent in the defibrinated blood, and Newton [1932] showed that such a toxic agent did develop in defibrinated blood on standing and suggested it was histamine.   The relative absence in the isolated perfused lung of the rather severe changes found in the heart-lung preparation is rather surprising.   It may simply represent a superiority of heparinised blood as against defibrinated blood; there is no histological evidence on this point.   Other possible factors are the negative pressure ventilation, the lower pulmonary venous pressure, and the absence of the heart in the isolated lung preparation.

Certain types of experimental lung damage in the intact animal give rise to histological changes similar to those found in the perfused lung.   Cannon, Walsh, and Marshall [1941] produced a local anaphylactic reaction in the lungs by intratracheal instillation of egg albumen in rabbits sensitised to egg albumen.   The early histological changes were œdema, with alveolar exudate and distension of perivascular lymphatics, together with some hæmorrhage into alveoli and perivascular lymphatic spaces.   Although they described the perivascular red cells as being confined to lymphatic spaces, it is evident from their photographs, which were only of arteries, that the condition was one of diffuse periarterial hæmorrhage similar to that found in the perfused lung.   They attributed the changes to increased permeability of the capillary endothelium permitting the escape of both plasma and red cells.

Lung irritant gases (chlorine, phosgene, etc.) cause acute œdema of the lungs and also damage to the bronchial epithelium.   Winternitz [1920] has described the lung changes in dogs exposed to chlorine, phosgene, and diphosgene.   In the early stages there was alveolar exudate, distension of periarterial and peribronchial lymphatics, œdema of the arterial wall and periarterial connective tissue, but there was no hæmorrhage.   He found, however, that dogs which had been gassed some months previously and apparently recovered, when killed by asphyxia, chloroform, or strychnine, developed periarterial hæmorrhages in the lungs at the time of death.   Winternitz thought that the hæmorrhage was "obviously the result of rupture of the vasa vasorum," and that the rupture was caused by a rise of blood-pressure during the agonal struggling or convulsions, the vessels having been weakened in some way as a result of the earlier gassing.   These hæmorrhages appear to be exactly the same as those found in the perfused lung.

In animals rapidly decompressed to a pressure of about one-sixth of an atmosphere and kept at that pressure in oxygen for about two hours, lung changes were constantly found [Trowell, 1943].   In rabbits

the changes were vascular congestion and œdema, with alveolar exudate, distension of periarterial lymphatics and œdema of arterial walls; occasionally there was slight alveolar hæmorrhage. In rats, guinea-pigs, mice, and monkeys, superimposed on the picture of congestion and œdema there was alveolar hæmorrhage and sometimes periarterial hæmorrhage. The precise cause of these lung changes was not discovered; it was considered that they were probably due to anoxia and circulatory changes rather than to mechanical air-pressure changes in the lungs. In blast injuries of the lungs (pulmonary concussion) the histological picture is quite different; in the early stages the sole finding is hæmorrhage, large interstitial hæmorrhages or more frequently small scattered alveolar hæmorrhages; there is no œdema or periarterial hæmorrhage [Zuckerman, 1941].

It is evident from the foregoing descriptions that the main pathological features of the perfused lung, alveolar exudate, periarterial lymphatic distension and periarterial hæmorrhage, are also met with in other types of lung damage, and collectively they seem to represent a common reaction of the lung to a variety of insults.

With regard to the cause of the œdema in the perfused lung, raised capillary blood-pressure is unlikely since the perfusion pressure was always less than the pulmonary arterial pressure of the intact animal, the venous pressure was generally zero, and histologically there was no sign of vascular congestion. The most likely cause, as in the case of other perfused organs, is increased capillary permeability due to some abnormality of the perfusing blood or to the absence of nervous capillary tone. Lymphatic obstruction must also be considered as a possible factor; when canulæ are tied in the trachea and the pulmonary artery some of the main lymphatics leaving the lung may be tied off. It is curious that in most of the types of experimental lung œdema only the periarterial, and not the peribronchial, lymphatics become distended. This is difficult to reconcile with the usual conception [Miller, 1937] that these two sets of lymphatics form a common anastomosing network throughout their course.

With regard to the periarterial hæmorrhage, although there is no evidence that it is true hæmorrhage as opposed to diapedesis, still the most likely cause seems to be rupture (probably capillary) of the vasa vasorum. As already indicated, certain histological evidence points to the periarterial connective tissue as a region of mechanical weakness in the lung. The possibility of rupture of the pulmonary capillaries of the alveoli of respiratory bronchioles or alveolar ducts with the blood tracking back in the periarterial zone seems to be excluded by the fact that blood was never seen round the very small arteries. If the blood does come from the vasa vasorum it must be remembered that these vessels are part of the bronchial circulation. The bronchial circulation was not directly perfused in these experiments, but it is

known from injection experiments that owing to the free capillary anastomosis between the bronchial and pulmonary systems in the region of the respiratory bronchioles and alveolar ducts, perfusion of the pulmonary circulation almost certainly results in a back-flow of blood into the bronchial circulation. It has been mentioned that in some experiments the bronchial vessels were congested; this indicates that such a back-flow did occur and that the pressure in the bronchial capillaries was fairly high due presumably to some obstruction of the veins draining the bronchial tree. It must be admitted, however, that periarterial hæmorrhage occurred quite as frequently in cases which showed no bronchial congestion. It seems that both in perfusion and other circumstances, periarterial hæmorrhage seems to go hand in hand with œdema and distension of the periarterial lymphatics. It may be that the hæmorrhage is caused by distension or rupture of these lymphatics.

SUMMARY.

A description is given of the histological changes found in the isolated perfused lungs of dogs. The perfusion was carried out with heparinised blood under negative pressure ventilation for $3\frac{1}{2}$ to 7 hours. The main findings were:

(1) Œdema: comprising alveolar exudate, distension of periarterial lymphatics and œdema of arterial walls.
(2) Periarterial and peribronchial hæmorrhage.
(3) Collections of polymorphonuclear leucocytes in the small pulmonary blood vessels.
(4) Dilation of bronchi and bronchioles.
(5) Vascular congestion of bronchial walls (except in experiments in which the outflow was allowed to drain from the open pulmonary veins).

The alveolar walls, bronchial epithelium and vascular endothelium were normal and there was no pulmonary vascular congestion. It is pointed out that changes (1) and (2) have been recorded in other types of experimental lung damage and they seem to represent a common reaction of the lung to various modes of injury. The possible causes of these changes are discussed.

The histological changes which have been described in the lung of the heart-lung preparation were for the most part not found in the isolated perfused lung.

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Photomicrographs of Paraffin Sections of Perfused Dogs' Lungs.

FIGS. 1–4.—Stained with Hæmalum and Biebrich Scarlet.
FIGS. 5, 6.—Stained with Hæmalum, Aurantia, and Aniline Blue.
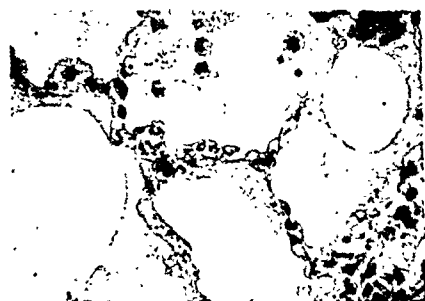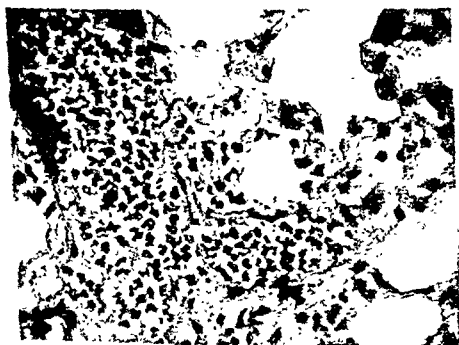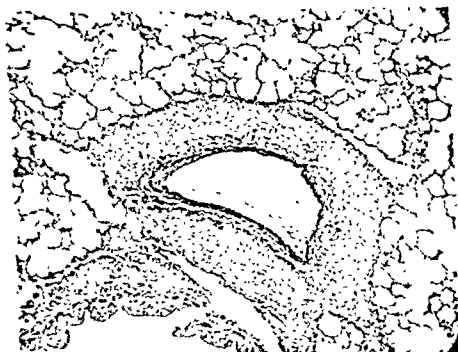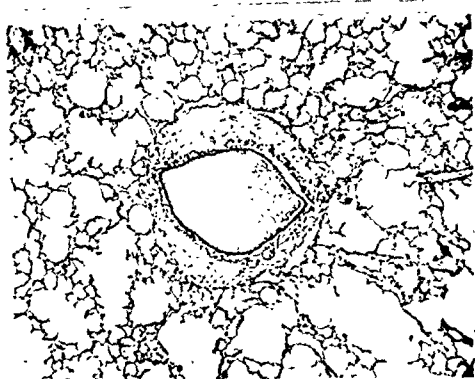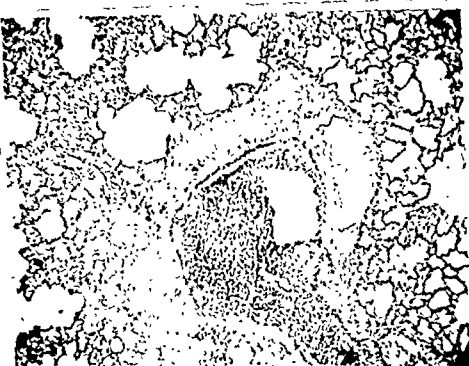
FIG. 1.— Alveolar exudate. Distension of periarterial lymphatics. (× 35).
FIG. 2.— Alveolar exudate. Œdema of arterial wall. (× 35).
FIG. 3.— Periarterial hæmorrhage. Distension of periarterial lymphatics. (× 35.)
FIG. 4.— Peribronchial hæmorrhage. (× 35.)
FIG. 5.— Polymorphonuclear leucocytes in a venule. (× 290.)
FIG. 6.— Polymorphonuclear leucocytes in capillaries. (× 290.)

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SOME MORPHOLOGICAL FEATURES OF THE SPINAL CORD IN THE RABBIT, WITH SPECIAL REFERENCE TO THE PRODUCTION OF ARTIFACTS. By O. A. TROWELL. From the Physiology Department, University of Edinburgh.

DURING an investigation which involved the post-mortem exposure, removal, and subsequent histological study of the normal spinal cord in most of the common laboratory animals, some rather peculiar artifacts were encountered in the case of the rabbit. The artifacts were produced during the exposure of the cord and were ultimately related to certain morphological peculiarities of the spinal cord and meninges in this species. These peculiarities of the rabbit's cord do not appear to be generally known and no record of them could be found in the literature, though it is difficult to believe that they can have escaped the attention of previous workers. This paper will describe the nature of the artifacts, their mode of production, and methods for their elimination. This knowledge is of particular importance in the pathological investigation of acute traumatic injuries of the spinal cord and it may prove of practical value to other workers.

I. EXPOSURE AND REMOVAL OF THE SPINAL CORD IN ANIMALS OTHER THAN THE RABBIT.

The spinal cord was exposed and removed by the following method, which, in principle, is the same as that employed at human autopsy. The posterior surface of the vertebral column was fully exposed by cleaning away the attached muscles. The vertebral spines and laminæ were then removed with suitable instruments. In the larger animals (cat, dog, goat, monkey) the laminæ can be cut off at their base with laminectomy forceps, but in smaller animals (rat, guinea-pig, rabbit) it is necessary to remove the spines and laminæ piecemeal with various types of nibbling forceps. Fortunately, in these animals the epidural space is relatively larger (and the subarachnoid space correspondingly smaller) than in man, and it is generally possible to open the vertebral canal in this way without damaging the dural sac or its contents. Having thus exposed the cord in its dural sac, either the nerve roots were cut with fine scissors and the whole cord, still enclosed in dura,

213

nerves leave the cord approximately at the levels of the corresponding vertebræ. The cervical, thoracic, and lumbar regions of the cord therefore correspond to the same regions of the vertebral column.

After fixing the cord *in situ*, histological sections through the
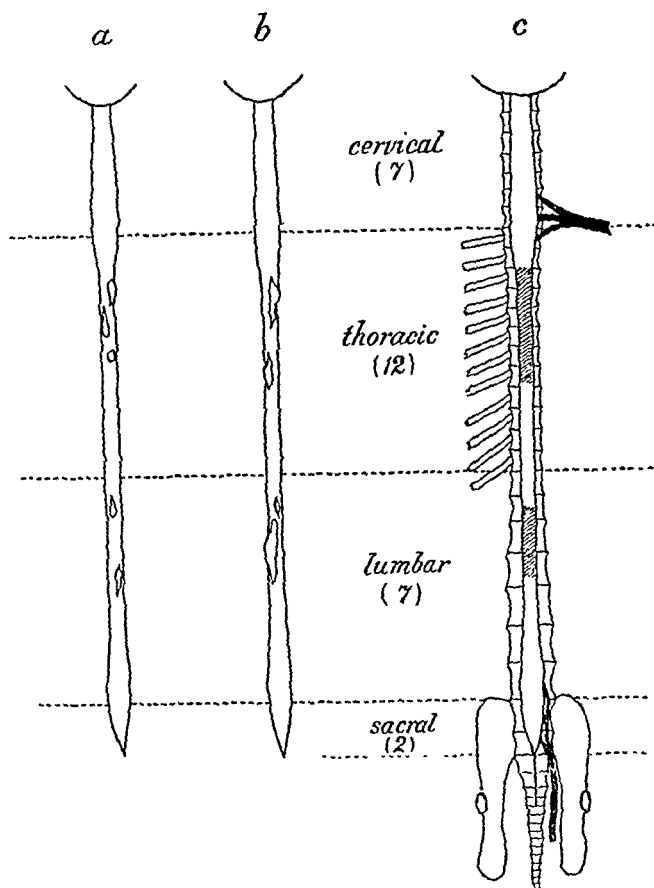
FIG. 2.—Semi-diagrammatic drawings of rabbit's cords.   (*a*) and (*b*) to show the size, number, and location of swellings in two typical cases; (*c*) to show the relation of the spinal cord to the vertebral column, the main origin of the limb plexuses, and the regions of softer consistence (shaded).

swellings showed that in addition to gross distortion of the shape of the cord there was, underlying each swelling, usually a cavity of considerable size (fig. 3). These cavities had ragged torn edges and obviously represented a rupture of the cord substance; they appeared to be a result of the external distortion rather than the cause of it. Examination of a large number of such cavities showed that usually the cavity originated as a horizontal cleft in the lateral grey matter on one or other side of the central canal (fig. 3, *b*, *c*). Thence the

lifted out and placed in formalin; or, alternatively, the whole animal was placed in a tank of formalin and the cord removed later after fixation *in situ*. The latter method was usually adopted, because it is generally believed that handling, especially bending, of the unfixed cord may produce nerve fibre artifacts, although in the cases in which the former method was used no such artifacts were in fact observed. This method, applied to normal animals killed with coal gas, was satisfactory in all the species (other than the rabbit) examined—rat, guinea-pig, cat, dog, goat, and monkey. The histological sections of the spinal cord were in all cases quite normal.

II. Exposure and Removal of the Spinal Cord in the Rabbit. Occurrence of Artifacts.

The rabbits used in this investigation were adults aged 1–3 years and weighing 1·6–3·5 kg. They were of mixed breed from a variety
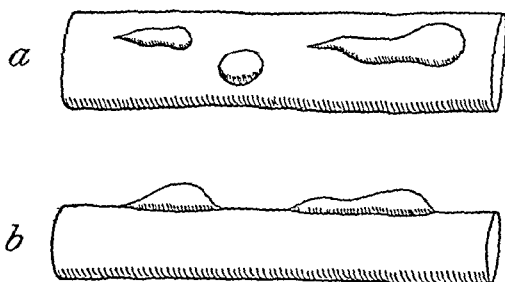
Fig. 1.—Swellings on the rabbit's spinal cord due to spontaneous herniation of cord substance through posterior dural incisions. (*a*) posterior aspect of cord, (*b*) lateral aspect.

of common strains. They were killed with coal gas. Examination of the fresh cord was completed and the cord placed in fixative (formalin) within two hours of death.

In 16 rabbits the spinal cord was exposed by the method described in I. above, the spines and laminæ being removed piecemeal with nibbling forceps. In the majority, but not all, of these animals, the cord after exposure presented a peculiar appearance in that there were a number of discrete swellings on its posterior surface. These varied from low fusiform swellings parallel to the long axis of the cord to hemispherical knob-like protuberances. With a few exceptions these swellings occurred only in two regions of the cord, the upper half of the thoracic region and the upper lumbar region. The appearance of these swellings is shown in fig. 1. Fig. 2, *a*, *b* indicates their size, number, and situation in typical cases. It should be mentioned that in the rabbit the spinal cord extends down to the level of the second sacral vertebra, and, except in the sacral and caudal regions, the spinal

ragged edges.. These involved both grey and white matter indiscriminately and had a very characteristic radial distribution (fig. 4). This appearance of multiple radial cracks will be referred to as the Type B artifact. Sometimes the Type B artifact was met with, superimposed as it were on the Type A artifact, in the region of a swelling, but very often it occurred elsewhere in regions of the cord which were quite normal to the naked eye. On the whole, however, it was most frequently found in those two regions of the cord which have already been mentioned as the sites of predilection for the Type A artifact. In addition to these specific appearances, the cord, throughout its length, showed a slight and variable distortion of shape; in transverse section it was never quite symmetrical (fig. 4).

III. The Cause of the Type A Artifact.

It was discovered that the Type A artifact represented a bulging or herniation of the cord substance through holes torn in the dura mater. The dura had been torn at several points by jagged bone fragments during the process of opening up the vertebral canal. This was by no means obvious at first sight, for in these animals the dura was a very thin transparent membrane, easily torn, and quite tightly applied to the posterior surface of the cord; indeed careful inspection was required to detect its presence at all. In other species, notably in man, the subarachnoid space is much larger and the dura is thicker, relative to the size of the cord.

That the swellings had been caused in this way was proved as follows. In the first place it was found that if more care was exercised in removing the vertebral arches it was possible to avoid tearing the dura. This simply involved working more slowly and removing the bone in very small fragments, the whole procedure taking an hour or more. If the dura was not torn, then there were no swellings. If now in such a cord the dura was picked up on the point of a scalpel and incised for a length of about 5 mm. without cutting or bruising the underlying cord, it was observed that the dural edges retracted and the cord slowly bulged through the hole, the swelling taking about 2 minutes to attain its full dimensions. A transverse dural incision opened out into a circular hole and produced a hemispherical swelling, while a longitudinal incision opened into a lens-shaped hole and produced a fusiform swelling. Very often, after the incision had been made, the dura tore further along the same line and so quite large holes eventually resulted from quite small incisions. Only in two regions of the cord, the upper thoracic and the upper lumbar, did dural incisions give rise to large swellings; elsewhere no, or only very small, swellings developed. By making incisions of varying size and direction in the two susceptible regions it was possible to reproduce all the varieties of

cavity might subsequently extend either horizontally outwards through the white matter (fig. 3, *g*, *h*) or else posteriorly along the line of the posterior horn (fig. 3, *d*, *e*, *f*), in either case frequently breaking through to
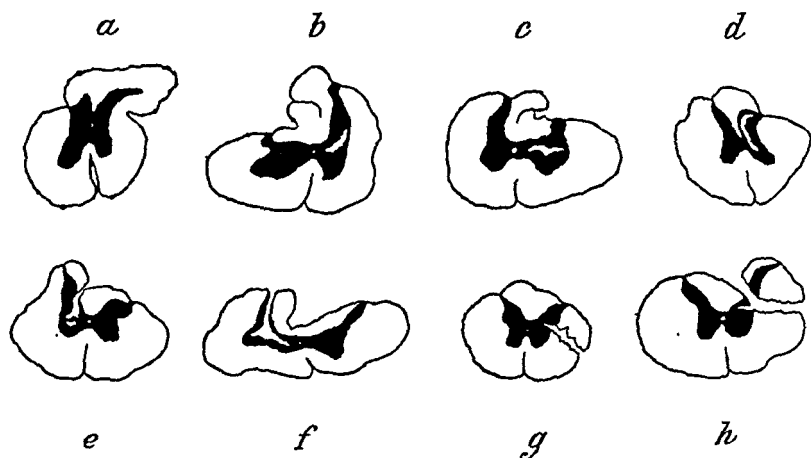
FIG. 3.—Transverse sections of rabbit's cords. The dura had been torn or incised at various points and the sections are taken through the resultant swellings. The Type A artifact.
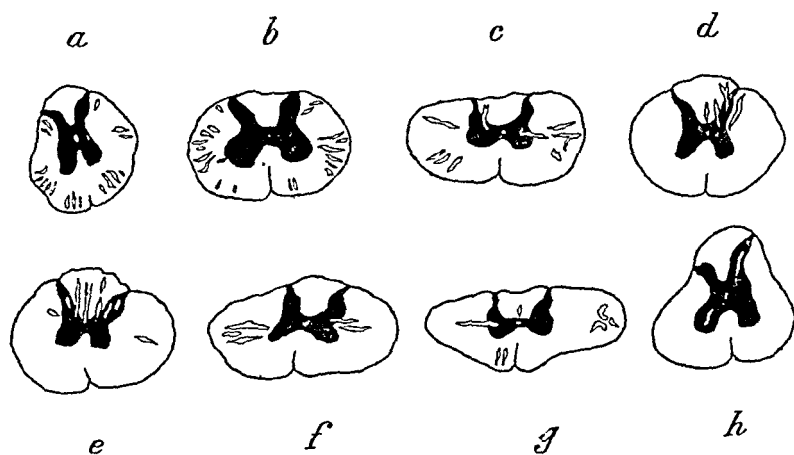
FIG. 4.—Transverse sections of rabbit's cords, fixed with the dura intact. The Type B artifact.

the surface. For convenience, these appearances—that is, the external swellings with the large underlying cavities—will be called the Type A artifact.

During the histological examination of the cords, another and quite different type of abnormality was met with in certain regions. This took the form of numerous small slit-like cavities, again with

size of the swellings which resulted from dural incisions depended on the attitude of the column. In full flexion the swellings were very large; in full extension no swellings developed and cerebrospinal fluid escaped freely from the incision, showing that the dural pressure was removed. Likewise, existent swellings could be made to vary in size, to disappear and reappear, by appropriate flexion or extension.

It should now be explained that for the exposure of the cord, the animal's shoulders were always raised on a block so as moderately (but not fully) to flex the upper part of the vertebral column; this gave better access to the laminæ and it was done also in the other species. The usual flexed position was therefore a contributory factor in the development of swellings in the upper part of the cord. Flexion and extension had, however, little or no influence on the size of swellings produced in the lower part of the cord.

Apart altogether from the swellings, the general shape of the cord was greatly altered by flexion or extension of the vertebral column. This was most marked in the lower cervical-upper thoracic region where the shape could be varied from that shown in fig. 4, *g* in flexion to that shown in fig. 4, *h* in extension.

The observations recorded in (i) and (ii) were confirmed in 7 animals.

(iii) *Soft Consistence of the Cord Substance.*—It had been noted that the cord substance which protruded through the dural incisions was remarkably soft in consistence. At first sight it seemed likely that this was due to bruising of the cord substance during exposure of the cord, but this was proved not to be the case. In 8 animals the cord was exposed very carefully so that bruising was absolutely excluded and the dura was intact. The dura was then cut down the middle line and reflected in the way previously described. The consistence of the cord was now carefully explored in all regions, feeling it with a blunt probe and noting its resistance to deformation. In the first place the impression was gained that the cord as a whole was definitely softer than in other species. This, however, was a difficult judgment to make and it will not be insisted on. The second discovery was quite definite, that in certain regions the cord was softer than elsewhere. In these soft regions the consistence could only be described as semi-fluid or pultaceous, the cord substance resembling a formless paste which could be poked into almost any shape by quite light pressure. Indeed in these soft regions, once the dura had been reflected, the cord had not sufficient rigidity to maintain its normal shape; sections of such regions fixed *in situ* always showed the cord markedly distorted and asymmetrical (fig. 5). Usually two such soft regions could be defined, one being roughly the upper half of the thoracic region, the other being in the lumbar region (fig. 2, c). The first of these two regions could always be easily defined, the transition from soft to "normal" cord was fairly sharp, and it was fairly constant in position from

Type A artifact which had been encountered in the original cords. Histological examination showed the same underlying cavities which evidently represented an internal rupture of the cord substance consequent on the herniation.

Given dural tears, therefore, herniation of the cord substance occurred spontaneously. This seemed to be a rather remarkable phenomenon and it called for further investigation. Ultimately, three morphological factors, all apparently peculiar to the rabbit, were found to play a part in bringing about this spontaneous herniation. These three factors will be dealt with in turn, and it must be emphasised that one is dealing here only with the morphology of the cord and its meninges as it presents itself post-mortem after opening the vertebral canal. In the living animal conditions may be different.

(i) *The Dura Mater is Tensely Stretched and Exerts, Pressure on the Posterior Surface of the Cord.*—From the way in which experimental dural incisions "opened out" it was obvious that the dura over the posterior aspect of the cord was tightly stretched and that the tension was greatest in the longitudinal direction. Also the way in which the cut edges sank into the cord and squeezed up cord substance in between made it clear that the dura was actually exerting pressure on the cord, the subarachnoid space being obliterated in this region (this was confirmed by the fact that no cerebrospinal fluid escaped from the incisions). Dissection showed that the dura was everywhere very close to the cord, but only on the posterior aspect was it actually pressing on the cord and obliterating the subarachnoid space. The arachnoid could not be defined by dissection but it was recognised histologically, little more than a double layer of mesothelial cells. It always remained attached to the dura and was of course divided whenever the dura was cut. The pia mater generally remained intact even over quite large swellings. Finally it was shown that if the dura was cut down the middle line and entirely reflected from the whole cord, then any swellings previously present as a result of local dural incisions now disappeared almost entirely. This proved conclusively that the primary cause of the swellings was the pressure of the dura on the cord. In these experiments the dura was opened by lifting it up off the cord on the point of a fine scissors, the scissors were then run along the whole length, keeping clear of the cord and so avoiding damage.

(ii) *Flexion of the Upper Vertebral Column Increases the Pressure of the Dura on the Cord in that Region.*—In the cervical and upper thoracic region (only) the pressure of the dura against the posterior surface of the cord is increased by flexion of the vertebral column and decreased by extension; in full extension the pressure is removed entirely. This region of the rabbit's vertebral column is very mobile, but the lower half (lower thoracic, lumbar, sacral) is almost immobile. In correspondence with this it was found that in the upper part of the cord the

dura must also have bruised the cord in the same regions. This may have been the case and may have contributed to the original artifacts, but it is not an essential factor for it has been shown· that swellings develop when the dura is incised over unbruised cords. Again, in the
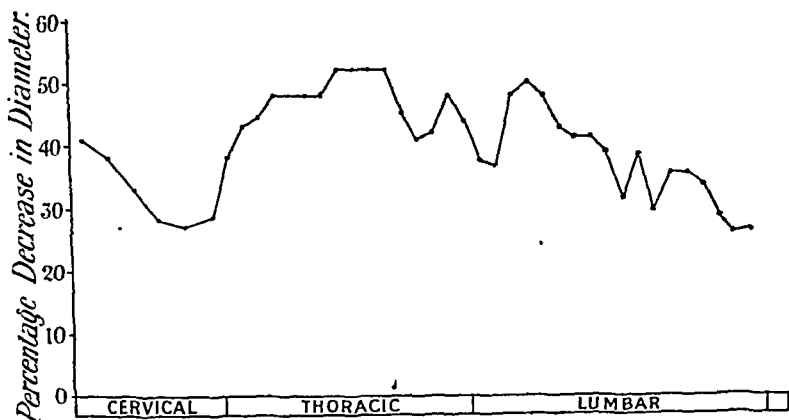
FIG. 6.—Deformation of a fresh rabbit's cord caused by the application of a constant weight, at the various levels. A lever was allowed to rest on the posterior surface of the cord and the percentage decrease in the antero-posterior diameter was measured.

original exposures the dura may have been torn only in those regions where swellings developed (it was not possible to verify this), but this also would be an unimportant factor for it has been shown that dural incisions elsewhere result in no, or only very small, swellings.

IV. The Cause of the Type B Artifact.

This artifact occurred most commonly in the two soft regions of the cord, occasionally elsewhere. It still occurred in cords which had been carefully exposed without tearing the dura, and then fixed *in situ* before removal. Such cords showed in addition some slight distortion of shape (fig. 4). If the dura was reflected before fixation the distortion of shape was more marked (fig. 5) and the Type B artifact was also present. It is worth mentioning that this distortion of shape was not met with to anything like the same degree in the cords of other species handled in the same way. This supports the view already advanced that the rabbit's cord is softer than that of other species. Evidently the Type B artifact was not associated in any way with the Type A, and it was not due to direct handling (*e.g.* removal) of the cord before fixation. It was found to be entirely eliminated if the cord was fixed *in situ* in the unopened vertebral canal—by methods to be described in the next section. This artifact was, therefore, produced in some way during, or as a result of, opening of the vertebral canal.

animal to animal.   The second and lower region was neither so constant
nor so definite.   It varied in position by as much as 2 cm. in different
animals and was usually less sharply defined, the transition from
"normal" cord being more gradual.   The consistence of the cord
between these two soft regions was somewhat variable from animal to
animal.   Usually it was slightly softer here than in the cervical and
lower lumbar regions, but nothing like so soft as the two soft regions
proper; in one or two cases, however, this part of the cord was quite
soft, and in these cases it was possible to recognise only one continuous
soft zone extending from Th. 2 to L. 4.   It should be mentioned that
the pia was always intact even in the soft regions and it was not torn

Fig. 5.—*Transverse sections of rabbit's cords, the dura reflected before fixation.*

when the cord was explored with the probe.   In the soft regions the
pia was very lax and "baggy," it appeared as a crinkled membrane
rather loosely applied to the cord substance; elsewhere it was tense
and closely applied.

In order to obtain some objective record of these soft regions of the
cord, a fresh cord was carefully removed, the dura reflected and the cord
then subjected to local pressure by allowing a lightly weighted lever
to rest across it.   The tip of the lever recorded on a scale, and by
observing its position, first with the lever just touching the cord, and
then with lever resting on the cord, the percentage reduction in diameter
of the cord following the application of a constant weight was obtained.
Observations were made serially along the cord and the results are
shown in fig. 6.   The morphological explanation of this softer con-
sistence of the cord in certain regions will be discussed later (Section VI).

These two soft regions appeared to correspond, more or less, to
those two regions of the cord in which it had been found that herniation
of cord substance most readily occurred, and this apparently explained
why the Type A artifact was chiefly found in these two regions in the
original animals.

While it must be admitted that the primary cause of the Type A
artifact was the tearing of the dura during the somewhat careless
exposure of the cord, a similar carelessness did not produce it in the
other species.   Given that the dura is torn, then it seems that the three
factors described above would account for the presence and the dis-
tribution of the Type A artifact in the original cords.   It might be
argued that in the original exposures damage sufficient to tear the

These results confirmed the fact that all the artifacts originally encountered were the result, direct or indirect, of exposing the cord before fixation.

VI. Morphological Factors which may Account for the Soft Regions present in the Normal Rabbit's Cord.

It seemed worth while to see if the soft regions differed histologically in any way from the rest of the cord. First of all five cords were exposed, the dura was reflected and the soft regions were localised by palpation and marked. After fixation, sections from soft and "normal" regions were compared. There were no obvious qualitative differences, and in particular the soft regions showed no signs of bruising or post-mortem change. Having thus excluded the possibility of disease, trauma, and post-mortem change, it seemed likely that differences, if any, must be in the relative proportions of the several structural components. This possibility was investigated as follows. A cord was fixed *in situ* by the Method 2 described in the preceding section. After fixation the cord was exposed and divided transversely into segments corresponding to the respective vertebræ; from Th. 10 downwards, the vertebræ being longer in this region, each vertebral segment was subdivided into two. Paraffin sections were prepared from the middle portion of each of the 33 pieces and subjected to a careful serial comparison. Sections were stained by (1) Nissl method, (2) Bodian silver method, (3) Mallory neuroglia method. Broadly speaking the findings were that the whole region from about the Th. 2 to L. 4 differed in several respects from the rest of the cord. This region corresponds to the two soft regions plus the intermediate (lower thoracic) part between them; it also corresponds to the region between the cervical and lumbar enlargements (fig. 2, *c*), and it seems likely that the differences found simply represent differences between the enlargements and the rest of the cord. The region Th. 2 to L. 4 differed from the rest of the cord in the following respects:—

1. *Proportionally less Grey Matter.*—Fig. 8 shows the areas of the total cord, grey matter and white matter at the various levels. The areas were measured by cutting out and weighing projection drawings. The ratio of grey to white matter in the cervical and lower lumbar regions was about 1:3, in the intermediate region it was about 1:6.

2. *Posterior Columns Relatively Smaller.*—Fig. 8 shows that the ratio of the posterior columns to the rest of the white matter was, in the cervical and lower lumbar regions, about 1:5, and in the intermediate region about 1:9.

3. *Average Size of Fibres Greater.*—In the region Th. 2 to L. 4 the white matter contained relatively more of the large-sized fibres and also these "large" fibres were themselves larger than the "large" fibres of other regions. Fig. 9 shows camera lucida drawings of comparable regions of the white matter at the levels C. 5, C. 6, and Th. 3, Th. 4. The region selected for drawing was in the lateral white matter midway between the grey matter and the lateral

The soft consistence of the cord evidently favoured its production, but its precise method of causation was not discovered. It is believed that the possibility of bruising was eliminated, and it is suggested that it may have been caused by flexions and extensions of the vertebral column after the cord had been exposed; the change in shape which the cord undergoes under these conditions has already been mentioned (III (ii)).

V. Methods for Fixation and Removal of the Rabbit's Cord without Artifact.

It was concluded that histological sections entirely free from artifact and distortion of shape could only be secured if the cord was fixed before opening the vertebral canal. Two methods were devised for doing this.

Method 1.—The vertebral column was removed entire and divided into five lengths by careful disarticulation between vertebræ, the cord and meninges
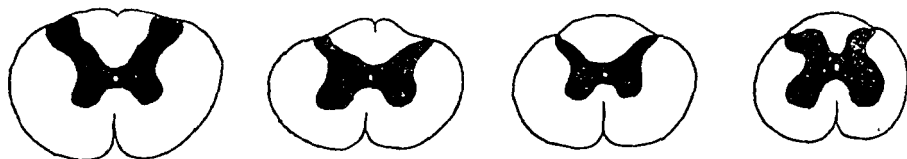
FIG. 7.—Transverse sections of rabbit's cords, the cord fixed *in situ* before opening the vertebral canal.

being cut cleanly with a razor blade. These lengths of column were fixed in 8 per cent. neutral formaldehyde and subsequently decalcified in 5 per cent. formaldehyde plus 10 per cent. nitric acid. When decalcification was complete, they were sectioned by the paraffin method, sections being taken only from the middle region of each length. Fourteen spinal cords were treated in this way and in all cases the shape of the cord was perfectly preserved and there were no gross artifacts. Owing to the acid treatment the cells and fibres were considerably shrivelled (weaker decalcifying solutions were found inadequate) and some of the standard staining methods (*e.g.* Nissl) were interfered with. This method was theoretically perfect in that handling of the cord (in selected regions) was entirely eliminated throughout, but it was unsatisfactory in that only selected regions could be studied and that the material was *useless for detailed histological examination*. The second method was free from these drawbacks.

Method 2.—The vertebral column was removed entire and thoroughly cleaned from muscle on all sides. It was fixed in strong (20 per cent.) neutral formaldehyde for a week. This effected quite satisfactory fixation of the cord. The vertebral canal was then opened in the ordinary way and the cord removed. Provided ordinary care was exercised (no acute bending), it was found that no harm resulted from handling of the fixed cord: *the important thing was to avoid both handling and exposure before fixation.* Two cords were treated in this way and the sections were satisfactory in all respects (fig. 7). This method can be recommended for any histological investigation of the rabbit's spinal cord.

largely accounted for by the fact that the small fibres are mostly intersegmental (propriospinal) fibres whose numbers are roughly proportional to the size of the grey matter at the various levels; they are therefore most numerous in the cervical and lumbar swellings, and least numerous in the region Th. 2–L. 4, where as already shown the grey matter is proportionately least.

4. *Proportionally more Myelin Present.*—A consequence of the greater average size of the fibres must be that there is relatively more myelin per unit area of white matter.   This is fairly obvious from fig. 9.

5. *Proportionally less Neuroglia Present.*—Since there is a neuroglial network round every fibre, however small, it seems likely that if the average size of the fibres is greater and there are therefore fewer fibres per unit area, there will be correspondingly less neuroglia per unit area.   This supposition was borne out on examination of the neuroglia stained sections, but it was found impossible to make any accurate quantitative comparison of the amount of neuroglia present at different levels.
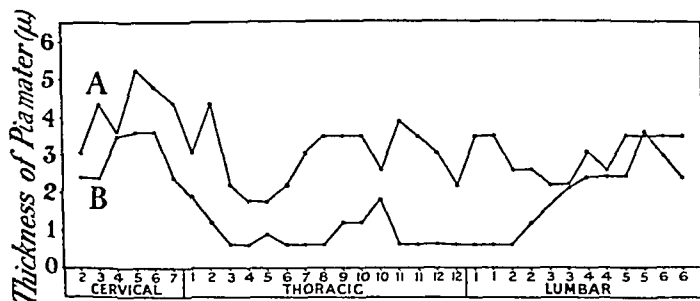
FIG. 10.—Rabbit's cord.   Thickness of the pia mater at the various levels.
A, in the anterior median fissure (double layer); B, on the posterior surface of the cord.

6. *Thinner Pia Mater.*—The thickness of the pia mater was measured with an eyepiece micrometer.   The measurements can only give a very rough approximation to the true thickness of the pia owing to the variable histological shrinkage, etc.   The pia was measured in two regions, on the posterior surface of the cord (curve B, fig. 10) and in the anterior median fissure (curve A, fig. 10). In these cords no anterior median "fissure" was ever seen, only a septum was found, the whole septum was measured and it included presumably two layers of pia mater.   The results showed a definite thinning of the pia corresponding fairly well to the upper soft region and some indication of a second thinning corresponding less well to the lower soft region.

The thinning of the pia was the only feature found which showed any direct correlation with the two soft regions.   All the other changes found were uniform from Th. 2 to L. 4, and although this corresponded to the upper limit of the upper soft region and the lower limit of the lower, they included also the supposedly "normal" region between the two.

In five other cords sections were examined from each of the soft regions and from two "normal" regions.   The sections were from known levels, they were measured in the same way, and the results fitted in entirely with those described above.   In each of these cases paraffin, frozen, and celloidin sections both transverse and longitudinal were made, and, in addition to the methods already mentioned, Bielschowsky and myelin staining were employed, but no further differences were revealed.

Taken as a whole these results would afford a rational explanation for the

periphery of the cord. It gives a fair sample of the features which were present in the whole of the anterior and lateral columns of white matter (the posterior
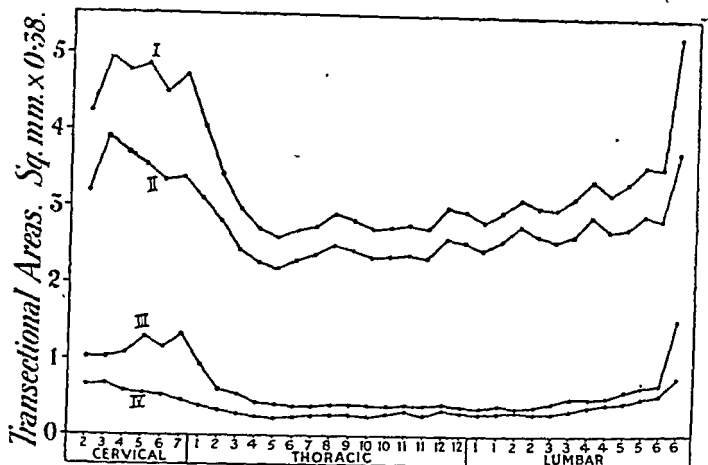
FIG. 8.—Rabbit's cord. Transectional areas of Total cord (I), White matter (II), Grey matter (III), and Posterior columns (IV) at the various levels.
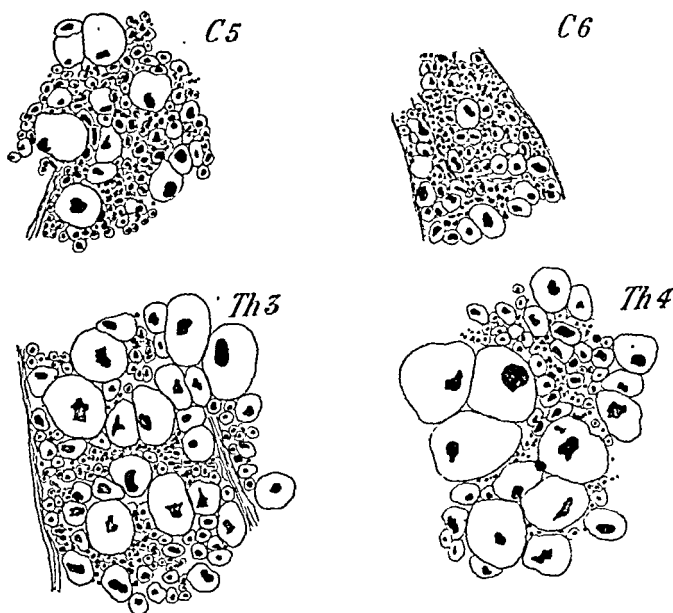

FIG. 9.—Rabbit's cord. Camera lucida drawings, all on the same scale, of comparable regions of the lateral white matter at the levels C. 5, C. 6 and Th. 3, Th. 4.

columns showed less difference). The change occurred fairly abruptly at the level Th. 2–3, it persisted uniformly down to about L. 4 and then the appearances reverted to those found in the cervical region. These differences can be

3–5 times the diameter of adjacent nerve fibres. They were met with from time to time in all regions of the white matter and at all levels of the cord, but they most frequently occurred in the posterior columns. They were also found in the brain of these animals, most commonly in the medulla and the subcortical cerebral white matter. They were never found in grey matter. The holes were always perfectly circular both in transverse and longitudinal sections of the cord; they were therefore not distended myelin sheaths. In longitudinal sections of the cord they often appeared in short rows (fig. 12, b) along the lines of the oligodendroglia cells and were very closely associated with these cells, apparently lying between them. They were present in paraffin, frozen, and celloidin sections, and were therefore not an artifact of paraffin technique. The edges of the holes were sharp and clear cut, but no organised wall could be defined, though they were embraced by a slight condensation of neuroglia fibres. They were clearly not empty distended capillaries, nor were they fat (Sudan IV staining) or myelin. No content of the holes could be revealed by any of the ordinary staining methods. They resembled in all respects the nitrogen bubbles which can be produced in the spinal cord by the sudden decompression of animals exposed to raised atmospheric pressure, except that these latter might attain a larger size. The same holes were also found in the brain and spinal cord of other species (goat, monkey), but they were more frequent in the rabbit. Neuropathologists appear to be unfamiliar with them in human material. It seems likely that they represent an artifact of some kind. Although they were found in material fixed within an hour of death, the possibility must be considered that they are due to some rapid post-mortem change. Ostertag [1931] describes the occurrence of cystic spaces in the white matter in the rabbit and other animals, which he ascribes to post-mortem swelling of myelin sheaths. It is probable that he was observing the same phenomenon as reported here and that these appearances have often been seen and dismissed as "swelling of myelin sheaths."

DISCUSSION.

The spinal meninges and meningeal spaces in the rabbit evidently differ somewhat from those in man, where the dura is a tough membrane separated from the cord by a fairly wide subarachnoid space. The only comparative account of the meninges in mammals appears to be that of Sterzi [1901] who states that the subarachnoid space is well developed only in man and the carnivora. In all the species examined here the spinal subarachnoid space appeared to be relatively smaller than in man; in the rabbit it was particularly small, its posterior part being entirely obliterated under post-mortem conditions. It is stated [Weed, 1922] that in all mammals the epidural space is filled with fat and veins.

whole region between the cervical and lumbar enlargements being of softer consistence than elsewhere, the most important factors probably being the proportionally greater amount of white matter and of myelin, and the proportionally less amount of neuroglia and pia mater. Apart from the rather unsatisfactory evidence regarding the thickness of the pia mater, they afford no explanation of the original description of two soft regions separated by a more or less normal region in between. As was pointed out originally, however, this intermediate region was variable from animal to animal, being in some cases almost as soft as the other two regions, and it may be that the animal selected for the serial investigation was such a case. On the other hand, the possibility is still open that the thickness of the pia mater really is the most important factor, but in order to decide this some more refined method of measuring the pia mater would have to be devised.

VII. ADDITIONAL HISTOLOGICAL OBSERVATIONS ON THE RABBIT'S CORD.

In the course of the histological examination of 42 rabbits' cords, two other features were observed which seem worthy of record. They were present in cords fixed before opening the vertebral canal and there was no reason to suppose that they were artifacts.

(i) *Variation in Size of the Central Canal.*—The central canal was found to vary in size at different levels in the same cord. Usually one
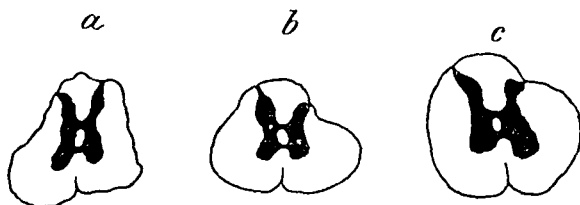
FIG. 11.—Transverse sections of rabbit's cords showing a wide central canal.
(a) Upper thoracic region; (b) mid-thoracic region; (c) lower cervical region.

considerably dilated region was found, where the diameter of the canal might be as much as one-eighth of the diameter of the cord. The position of this region varied from animal to animal; it might be in the upper cervical, the thoracic or the lumbar region (fig. 11). Most commonly it was in the upper thoracic region. The exact extent of these dilated regions was not determined. It is well known that many mammals show a ventricle-like enlargement of the posterior end of the canal (ventriculus terminalis), and Ostertag (1931) states that in the rabbit such an enlargement extends caudally from the level of the second lumbar vertebra. In the rabbits examined here, however, similar enlargements were found at other levels of the cord.

(ii) *Presence of Spherical Holes in the White Matter.*—In nearly all the cords examined a few circular holes were observed in the white matter (fig. 12). They were generally 15–30μ in diameter, or roughly

produced in the human cord. Van Gieson [1892] has described and figured artifacts produced during post-mortem removal of the human cord, but they were, as he admits, the result of gross damage inflicted with bone forceps and chisel. They mostly took the form of curious telescopic displacements of grey and white matter without much alteration of the external shape of the cord and without rupture of the dura, and they were accompanied by histological evidence of pulping of the cord substance. Such appearances were never met with in the work reported here. In addition to these common artifacts, van Gieson describes, as occurring less frequently, a herniation of cord substance through holes torn in the dura, the final picture resembling closely the Type A artifact of the rabbit. It is clear, however, that this artifact was only produced when cord substance was forced out of a dural tear by compression of the nearby cord with bone forceps; whereas in the rabbit herniation occurs spontaneously and in the absence of bruising. A survey of the literature on acute traumatic lesions of the spinal cord due to indirect violence shows that some of the so-called lesions may have been artifacts produced during removal of the cord. In man a condition variously labelled as Concussion of the spinal cord, Commotio Spinalis, Rückenmarkserschütterung, has been described as resulting from direct blows on the vertebral column, sudden flexions or extensions of the column, gunshot wounds, and blast from explosions [Holmes, 1915; Claude and Lhermitte, 1915; Davison, 1940]. The condition has also been produced experimentally in animals [Schmauss, 1890; Mairet and Durante, 1917]. The descriptions of the acute pathology of this condition have been very various and have included hæmorrhages, rupture of the cord substance producing cavities, and minute changes in the cells and fibres. Apart from the hæmorrhages, many of the changes described might have been artifacts produced during removal of the cord. In all cases the cord appears to have been removed before fixation. Holmes [1915] described as one of the histological findings in spinal concussion the presence of spherical empty vacuoles in the white matter. From his description and illustrations these appear to be indistinguishable from the spherical holes described here in the normal rabbit (Section VI (ii)). Davison [1940] shows photographs of spinal cord sections from cases of spinal concussion, some of which very closely simulate the appearance of the Type A artifact in the rabbit and others the Type B artifact. It may be, of course, that violence however applied, whether in life or during post-mortem removal, results in the same type of damage, the appearance merely representing the lines of mechanical weakness in the cord. Nevertheless it would seem important in all such work that the possibility of artifacts should be entirely eliminated and this can only be ensured by fixing the cord in situ in the unopened vertebral canal by some such method as that described in Section V.

In the rabbits examined here, a few veins were found on the surface of the dura, but the epidural space contained neither fat nor connective tissue. After opening the vertebral canal the epidural space appeared relatively large and contained only air. It is unlikely that this is the state in the intact animal. It is also unlikely that in life the dura exerts pressure on the posterior surface of the cord, or that the cord undergoes such marked changes in shape during flexion and extension of the vertebral column as were found post-mortem. One is led to the conclusion that in the intact rabbit the dura must be held off the cord in some way and the epidural space largely obliterated. How is this achieved? There were no fibrous connections of any sort between the posterior surface of the dura and the walls of the vertebral canal (there were a few fibrous strands anteriorly). Perhaps in the rabbit the epidural space, like the pleural cavity, is a potential space revealed only when it is opened and air allowed to enter. Or perhaps the pressure of cerebrospinal fluid keeps the dura distended and in contact with the walls of the vertebral canal. In the dog, Flexner, Clark, and Weed [1932] found that postural changes in cerebrospinal fluid pressure caused distension or collapse of the spinal dural sac with corresponding changes in the volume of the epidural space. As against either of these two explanations, however, it must be pointed out that at post-mortem examination the dura appeared to be inelastic and incapable of further distension without rupture.

In Section III it was shown that the Type A artifact was a consequence of certain morphological features of the cord and meninges in the rabbit. The fact that the artifacts never occurred in the other species suggests that those features are to some extent peculiar to the rabbit. It cannot be claimed, however, that any of the features described are entirely specific to the rabbit, because the other species were not carefully examined from this point of view.

Ostertag [1931] has described a method of exposing the brain and cord of the rabbit followed by fixation in situ, which seems to be essentially the same as the one used here. He used small bone forceps for removing the vertebral arches and the vault of the skull. He mentions, in respect of the brain, that tearing of the dura must be avoided as the brain substance readily "pours out" and this leads to artifact appearances, but he mentions no such occurrence in the spinal cord. Recently Falconer and Russell [1942], operating on living rabbits, found that removal of a small area of the vault of the skull together with the dura was followed by marked herniation of the brain and the development of cystic cavities in the underlying brain substance. It seems, therefore, that this herniation through dural incisions may be a peculiar property of the whole central nervous system in the rabbit.

There is some evidence that under certain conditions, probably differing from those prevailing here, somewhat similar artifacts may be

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In view of the widespread incidence of epizootic encephalomyelitis in rabbits, a disease which may produce softening and cavitation of the cord, it should be mentioned that none of the cords examined here showed any inflammatory changes.

SUMMARY.

1. The occurrence of artifacts in the spinal cord of the rabbit as exposed by the usual post-mortem methods is described. Such artifacts were not met with in the spinal cords of rat, guinea-pig, cat, dog, goat, or monkey, exposed in the same way. The artifacts occurred principally in the upper thoracic and upper lumbar regions, and were of two varieties—

(A) Large discrete swellings on the posterior surface of the cord associated with large underlying cavities. This artifact was due to spontaneous herniation of cord substance through small accidental tears in the dura mater. The spontaneous herniation was shown to be the combined result of three morphological features of the exposed cord and meninges in the rabbit, namely

(i) The posterior part of the dura is tightly stretched (mainly longitudinally) and exerts a direct pressure on the posterior surface of the cord, the subarachnoid space in this region being obliterated.

(ii) In the upper part of the spinal cord this dural pressure is accentuated by the usual flexed attitude of the upper part of the vertebral column at post-mortem examination.

(iii) In those two regions of the cord where the artifacts most commonly occur the cord substance is softer in consistence than elsewhere. Serial sections of the cord revealed quantitative differences in the proportions of the white matter, myelin, neuroglia, and pia mater at different levels which might account for the softer consistence in these two regions. The rabbit's cord as a whole appeared to be softer than that of other species.

(B) Multiple small radial cavities or cracks in the cord substance. These were shown to result from the exposure of the cord and to be related to the soft consistence of the cord, but their precise cause was not determined.

2. Methods are described for histological fixation of the cord without opening the vertebral canal, by which means the above artifacts can be eliminated.

3. Mention is made of the varying size of the central canal at different levels in the rabbit's cord.

4. The frequent occurrence of a few small spherical empty holes in the white matter of the rabbit's brain and spinal cord is reported. Their nature is discussed, but no conclusion is reached as to their significance.

5. The possible occurrence of similar artifacts in other species is discussed and the importance of eliminating such artifacts in all pathological investigations of acute traumatic lesions of the spinal cord is pointed out.
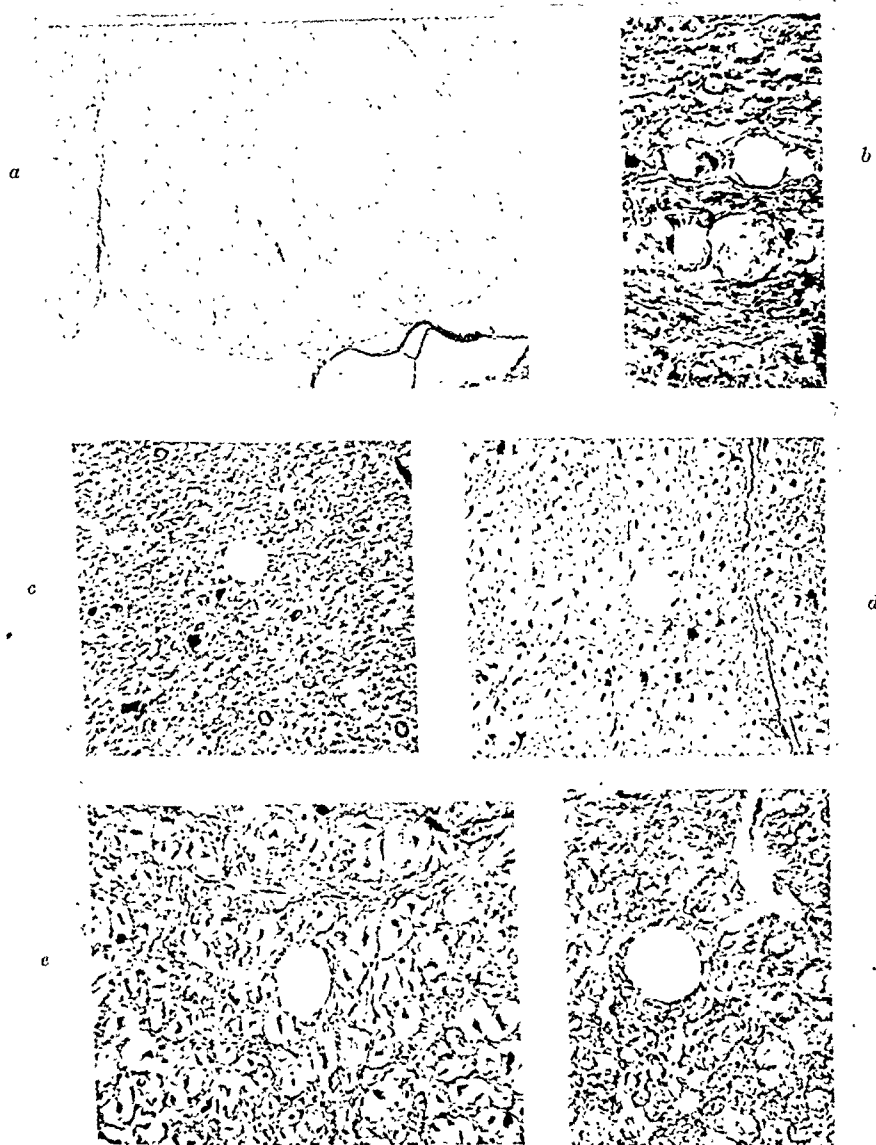
FIG. 12.—Spherical holes in the white matter of the rabbit's central nervous system.

    (a) Anterior column of spinal cord.   Transverse paraffin section.   × 60.
    (b) Posterior columns of spinal cord.   Longitudinal frozen section.   × 460.
    (c) Cerebral white matter.   Paraffin section.   × 300.
    (d) Anterior column of spinal cord.   Transverse paraffin section.   × 300.
    (e) Posterior column of spinal cord.   Transverse paraffin section.   × 460.
    (f) Posterior column of spinal cord.   Transverse frozen section.   × 460.

THE BEHAVIOUR OF CHOLESTEROL AND LECITHIN TOWARDS NORMAL HÆMOLYTIC SERUM.[1] By JUI-SHUAN LEE and CHIAO TSAI. From the Department of Physiology, College of Medicine, National Central University, Chengtu, China.

IN a previous communication [1942] we have reported that the hæmolytic action of dog serum towards rabbit cells can be inhibited by cholesterol. We have since extended our observations to sera and cells of a number of other species. In the meanwhile we have also performed experiments specially designed to elucidate the mechanism involved in this action. The results thus obtained are reported in this paper.

METHODS.

For a qualitative study we have employed sera and cells from a variety of animal species. The blood from ox and hog was procured fresh from abattoirs, kept cold, and used within 3–6 hours, while those from other animals were obtained in the laboratory either by vena puncture or by heart puncture and used within 2–3 hours. Serum was separated off from the clot by centrifugation and the cells washed three times with normal physiological saline and finally suspended in isotonic phosphate buffered saline (pH 7·30). Cholesterol suspension was prepared as described previously [Lee and Tsai, 1942].

The hæmolytic potency of each serum was tested in each experiment beforehand in order to gain an idea as to the amount of serum required for producing 50–100 per cent. hæmolysis. The anti-hæmolytic activity of cholesterol was then determined in a series of tubes, each containing 0·03 ml. of 50 per cent. washed cell suspension, a definite quantity of hæmolytic serum and a varying amount of cholesterol and of isotonic buffered saline, making up to a final volume of exactly 1·5 ml. The system was next placed in the water-bath at 37° C. for 30 minutes and then centrifuged. The percentage of hæmolysis was determined colorimetrically by matching the test sample with a series of standards prepared by lysing the same amount of cell suspension in water up to

though the degree of inhibition varies somewhat with the sera of different species. Since our data were obtained from a small number of cases (with the exception of dog and rabbit, only 2–5 animals from each species), it is not justifiable to make a quantitative comparison at present. But the impression we gained was that the anti-hæmolytic potency of cholesterol is lowest against goat serum, only moderate against sheep serum, and extremely variable with rabbit serum.

The serum of rabbits varied according to the breed. The serum from the albino rabbit of local strain is non-hæmolytic to the washed dog cells. We have tested more than 30 rabbits and observed only two cases exhibiting feeble lytic action. On the other hand, 4 out of 5 of the local black variety, and 9 out of 11 of the hybrids between the white variety and the Angora strain, show high hæmolytic action on dog cells. We have also tested 4 Angora rabbits of original strain and found only 2 of them possessing hæmolytic action on dog cells.

The action of cholesterol on the hæmolytic rabbit serum towards dog cells is exceedingly variable; the hæmolytic action of the black rabbit serum is not inhibited, while in 8 cases of the hybrids tested 3 show positive and 5 negative results. Cholesterol is not inhibitory or only slightly inhibitory to the hæmolysis of guinea-pig cells caused by local white rabbit serum.

2. *The Anti-complementary Action of Cholesterol and its Reversibility.*—If fresh dog serum is first mixed and incubated with a sufficient quantity of cholesterol, its hæmolytic activity toward rabbit cells is lost entirely. To demonstrate the anti-complementary action of cholesterol, it is first necessary to determine the exact amount of cholesterol that just completely annihilates the hæmolytic action of a definite quantity of serum. These amounts may be referred to as balanced quantities of cholesterol and fresh serum. The latter may be called cholesterol-inactivated serum. It behaves exactly like the heated serum. This is illustrated by the data given in Table II.

TABLE II.—SHOWING THE ANTI-COMPLEMENTARY ACTION OF CHOLESTEROL.

	Fresh serum required for 50 per cent. hæmolysis, μl.
1. Fresh serum alone	92
2. Fresh serum + 0·1 ml. heat-inactivated serum . .	50
3. Fresh serum + 0·1 ml. cholesterol-inactivated serum .	50

It will be seen that the hæmolytic potency of the fresh serum is increased by adding 0·1 ml. heat-inactivated serum, presumably due to the activity of amboceptor in the latter (compare 1 with 2). The

the same volume as in the test series, and rediluting each sample with a different amount of water. The minimum quantity of cholesterol suspension that produced a definite inhibition of hæmolysis was taken as a standard for comparison.

For the study of the mechanism of the anti-hæmolytic action of cholesterol only dog serum and rabbit cells were used. The amount of cholesterol used for each system of tubes was kept constant, *i.e.* always 0·1 ml. of 0·05 per cent. suspension, while that of hæmolytic serum was varied from 0·01 to 0·1 ml. in accordance with its hæmolytic potency. In order to see whether cholesterol acts on the complement or the amboceptor, the hæmolytic serum in some cases was heated to 50°–55° C. for 30 minutes before adding to the system. The hæmolytic potency of fresh dog serum was expressed in terms of microlitres (μl.) required for the production of 50 per cent. hæmolysis, and the anti-hæmolytic potency of cholesterol in terms of μl. of fresh dog serum inactivated by it. For the exact mode of calculation reference must be made to our previous report [Lee and Tsai, 1942].

RESULTS.

1. *The Anti-hæmolytic Action of Cholesterol towards Sera of different Animals.*—The results of this series of experiments are summarized in Table I, where the number of plus signs denotes the degree of inhibition; when the minimum effective amount of cholesterol is below

TABLE I.—THE INHIBITORY EFFECT OF CHOLESTEROL.

Serum.	Cell.						
	Rabbit.	Hog.	Dog.	Goat.	Sheep.	Guinea-Pig.	Man.
Man.	+ + +	..	..	..	..	+ + +	..
Ox .	+ + +	..	..	..	..	+ + +	..
Sheep	+ +	..	..	..	..	+ +	+ +
Goat	+	..	..	..	..	+	+
Dog.	+ + +	+ + +	..	+ + +	+ + +	+ + +	+ + +
Hog.	+ + +	..	+ + +	..	..	+ + +	..
Rabbit	..	..	+ + + or 0	..	..	+ or 0	..

·05 mg. in the 1·5 ml. system, we designate it with the symbol of + + +; when between 0·05–0·2 mg., + +; when above 0·2 mg., +; when 1·5 mg. still cannot produce visual effect, we consider it as no action (0).

It is noted that, excepting rabbit serum, the hæmolytic action of the other sera upon a variety of cells can all be inhibited by cholesterol,

amboceptor and complement, but preferentially inactivates the latter. When complement is present in the system, lecithin does not react with amboceptor. This is shown in Table IV. It shows that 35 μl. of fresh serum is inactivated when lecithin, heated serum, and fresh serum were mixed and incubated for 120 minutes (1). But if lecithin and heated serum were incubated for 118 minutes before being mixed with

TABLE IV.—THE EFFECT OF LECITHIN ON AMBOCEPTOR AT 24° C.

		Time of incubation.	Fresh serum inactivated, μl.
1.	Lecithin Heated serum } mixed Fresh serum	120 min.	35
2.	Lecithin Fresh serum } mixed	118 min., then heated serum added.	40
3.	Lecithin Heated serum } mixed	118 min., then fresh serum added.	20

fresh serum, the amount of inactivation was much lower (3). This may be attributed to the reinforcement of the amboceptor action. Nevertheless, when lecithin and fresh serum were incubated for an equal length of time and then mixed with heated serum (2), the degree of inactivation approached that in 1, indicating very little lecithin-amboceptor reaction taking place in the system. The anti-complementary action of lecithin is also reversible. It has been demonstrated by similar experiments to those tabulated for cholesterol. For the sake of brevity all the details and numerical presentation are omitted.

SUMMARY.

1. Cholesterol is able to inhibit the hæmolytic action of normal serum from a large number of mammalian species towards a variety of mammalian red blood cells. The only exception was found in the sera of some rabbits.

2. The sera of most of the local rabbits does not exhibit lytic property against dog cells; only that of a few, especially of the black variety, possesses this lytic action. The lytic activity of the sera of some of these rabbits is inhibited by cholesterol, but that of others is not.

3. This inhibitory action of cholesterol towards normal hæmolytic serum is due to the inactivation of the complement. Similar behaviour has also been observed with lecithin. Unlike cholesterol, lecithin is able

same degree of amboceptor activity, however, was observed when the added serum was inactivated by just sufficient cholesterol instead of by heat (compare 2 with 3). This indicates that the complement must have been knocked out of action and that the amboceptor remains unaffected by cholesterol.

It is of more importance to record that the anti-complementary action of cholesterol is reversible; *i.e.* when the complement is destroyed, the anti-complementary activity of cholesterol may be quantitatively recovered. As revealed in Table III, when just balanced quantities of

TABLE III.—REVERSIBILITY OF CHOLESTEROL ACTION ON COMPLEMENT.

	Procedure.	Fresh serum inactivated, μl.
1. Cholesterol ⎱ of balanced Fresh serum ⎰ quantity mixed	Kept at 37° C. for 30 min., then cooled to room temp. for 20 min. Fresh serum added finally.	0
2. Cholesterol ⎱ of balanced Fresh serum ⎰ quantity mixed	Kept at 37° C. for 30 min., heated to 52° C. for 15 min., and then cooled to room temp. for 5 min. Finally fresh serum added.	42

3. Cholesterol ⎱ of balanced
  Fresh serum ⎰ quantity treated
      separately.
	Each kept at 37° C. for 30 min., heated to 52° C. for 15 min., and then cooled to room temp. for 15 min. Finally they were mixed and fresh serum added.	43

cholesterol and of the fresh serum (column 1) are mixed, they are mutually inactivated; *i.e.* the former loses its anti-hæmolytic action and the latter its hæmolytic. If a further quantity of fresh untreated serum be added to such a system, no inactivation takes place (see 1). However, if the cholesterol-serum system is heated to 52° C. for 15 minutes, the process of inactivation of additional serum by cholesterol reappears (see 2). Similarly, if the serum of a balanced quantity is previously inactivated by heat and then mixed and incubated with cholesterol, the latter exerts a full anti-hæmolytic strength on the addition of fresh serum (see 3). The conclusion one would inevitably reach is that the action of cholesterol on the complement must be reversible; *i.e.* cholesterol is released and made available for further anti-hæmolytic action when the complement is entirely destroyed.

3. *The Action of Lecithin.*—The effect of lecithin is somewhat different from that of cholesterol. Lecithin reacts with both

THE BEHAVIOUR OF CHOLESTEROL TOWARDS HÆMOLYTIC IMMUNE SERUM.[1] By JUI-SHUAN LEE and CHIAO TSAI.

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SINCE cholesterol is able to inhibit the hæmolytic action of normal serum, it is interesting to see whether it possesses the same property towards hæmolytic immune serum. Our observations reported below have proved it to be the contrary. The absence of the anti-hæmolytic action of cholesterol in the immune serum may be due to the characteristic property of the acquired sensitizer or to the development of a special anti-cholesterol factor during immunization. The present experiments are designed to elucidate this point.

METHODS.

Rabbits, goats, and dogs were immunized with repeated intravenous administrations of washed dog cells, dog cells, and rabbit cells respectively. The procedure was carried out under strictly aseptic conditions. The dosage for each injection was usually between 0·5–1·0 ml./kg. of 50 per cent. cell suspension. The method of preparing cholesterol suspension, and that of determining the degree of hæmolysis, were as described previously [Lee and Tsai, 1942]. The hæmolytic potency of the immune serum was estimated in terms of hæmolytic units (H.U.) contained in 0·1 ml. serum. The quantity of serum that just caused 50 per cent. hæmolysis was estimated each time, and taken as 100 H.U. Knowing this, the H.U. in 0·1 ml. serum can be calculated according to the ratio of this amount of serum to that producing 50 per cent. hæmolysis. The anti-hæmolytic potency of cholesterol was expressed in terms of H.U. inactivated by it. If cholesterol reinforces instead of inhibiting hæmolysis, it should reduce the amount of serum required for the production of 50 per cent. hæmolysis; from the reduced quantity of serum we could compute the cholesterol hæmolytic units, which was prefixed with a minus sign in numerical presentation.

to reinforce amboceptor activity, but this occurs only in the absence of complement.

4. The inactivation of complement by cholesterol and by lecithin are both reversible; *i.e.* when the complement is destroyed by heat, the anti-hæmolytic potency of cholesterol and of lecithin is recovered quantitatively.

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LEE, J. S., and TSAI, C. (1941).   *Proc. Chinese Physiol. Soc. Chengtu*, Br. 1, 16.
LEE. J. S., and TSAI, C. (1942).   *Ibid.* 1, 69.

lytic action also could not be inhibited by cholesterol. This peculiar behaviour of rabbit serum is exceedingly interesting because it shows that the normal hæmolytic rabbit serum cannot be acted upon by cholesterol. Repeated injections of dog cells into these rabbits did not bring forth any change in the reaction of their sera towards cholesterol, in spite of a considerable increase of hæmolytic strength of the sera.

2. *Experiments with Goats.*—Normal goat serum is not hæmolytic to dog cells, but becomes so only after immunization with the latter. Five goats were employed in this series of experiments. Three goats were injected with dog cells once every 2 or 3 days for about one month, while the other two were given at first three injections at intervals of 2–4 days and then left for about one month, at the end of which five more injections at corresponding intervals were given. For convenience, we may call the former procedure "regular sustained injection" and the latter "series interposed injection." Tests for the hæmolytic activity of the sera and for the anti-hæmolytic potency of cholesterol were carried out from time to time throughout the entire period of experimentation.

The acquired hæmolytic property of goat sera can be easily demonstrated after one or two injections of dog cells. With regular sustained injection, the rate of acquisition of the sensitizers varied enormously with individual animals. In two cases the hæmolytic potency increased steadily with the number of injections, and continued to rise for many days even after cessation of the antigen administration. In the other case it remained stationary for a long time despite repeated injections, but began to rise progressively after the last injection and continued to rise for more than a month later. In all three cases the sera commenced to show resistance to the inhibitory action of cholesterol after the fifth injection. This tendency became stronger and stronger as immunization progressed until the anti-lytic action of cholesterol was completely prevented. The last condition persisted for a considerable time, depending upon the number of injections. With a greater number of injections the recovery of the cholesterol action took place much later. Fig. 2 shows the characteristic trend of the hæmolytic strength of the serum and that of the anti-hæmolytic potency of cholesterol during and after the antigen administration. The curves are plotted from the data of a goat under prolonged observation. One notes that the development of the anti-cholesterol property of the serum does not run exactly parallel with the increase of the hæmolytic strength. For instance, in the case presented in fig. 2 the abolition of cholesterol action occurs long before the hæmolytic strength has reached its maximum, and the recovery of the former commences at the time when the latter is still at its height. Unless we assume that the immune sénsitizer possesses a new property of annihilating cholesterol action, we cannot escape the conclusion that there develops in

RESULTS.

1. *Experiments with Rabbits.*—In the preliminary experiments we used both the intravenous and intraperitoneal routes for the administrations of washed dog cells. But we soon found that with intraperitoneal injection the development of immune hæmolytic power of the serum was so slow and irregular that we had finally to abandon it. Fifteen albino rabbits of local strain were used. In 13 of them the serum before immunization was non-hæmolytic to dog cells, whereas in the remaining 2 it was hæmolytic to it. The animals were all injected with washed dog cells three to four times at intervals of 3–14 days.
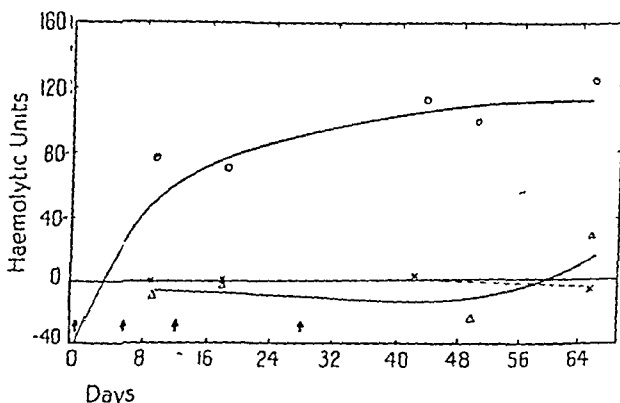
FIG. 1.—The hæmolytic potency (o-o-o) of the immune rabbit serum and the anti-hæmolytic potency of cholesterol (x-x-x, with 0·075 mg. cholesterol; and ↑-↑-↑, with 1·5 mg.) in a rabbit (No. 219) immunized with dog cell.

The hæmolytic strength of the serum and the anti-hæmolytic potency of the cholesterol were then estimated from time to time during the period of antigen administration and subsequently. The longest observation we have made was 38 days after the last injection.

The rabbit serum developed an immune hæmolytic action against dog cells quite readily. It occurred shortly after the first or second injection. In most cases the hæmolytic strength attained its height after the third injection and remained very strong for a considerable time. In all the 13 sera which had acquired the immune hæmolytic property, cholesterol in various concentrations from 0·075 to 3·6 mg. in the 1·5 ml. system invariably failed to prevent the hæmolysis of the dog cells induced by the immune sera; in a few cases it even tended to reinforce hæmolysis. Fig. 1 gives a typical picture showing that cholesterol in low concentration (0·075 mg.) exerts no inhibitory action, while in higher concentration it reinforces hæmolysis.

We have stated that the sera from 2 of the 15 rabbits used in this series was able to lyse dog cells before immunization. This natural

in the hæmolytic strength of the serum and in the anti-hæmolytic
potency of cholesterol. It is seen that the rise and fall of the two
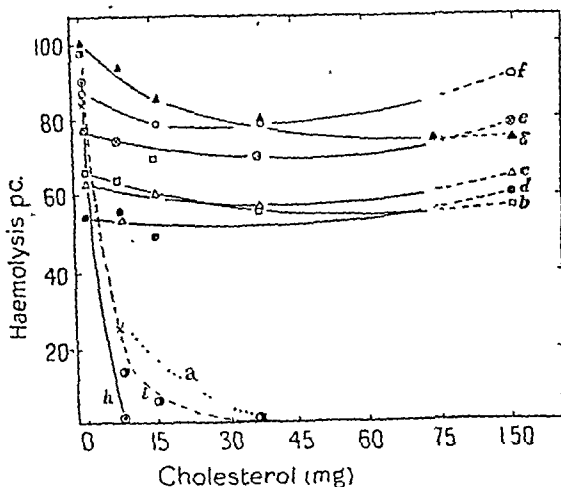
FIG. 3.—Graphs plotted from the same data as in fig. 1, showing the relation
of the anti-hæmolytic activity of cholesterol and its concentration in the serum
taken at different stages of immunization and afterwards. Curve (a) from the
initial stage; (b), (c), (d), (e), (f), (g) from subsequent stages; (h) and (i) about
one and two months after the last injection respectively.
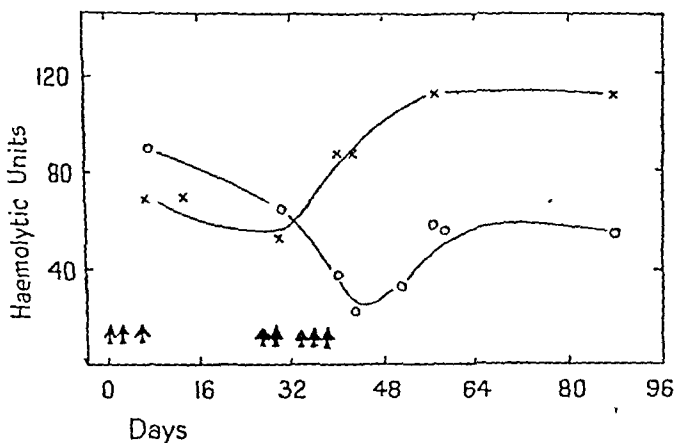
FIG. 4.—Graphs showing the hæmolytic (x-x-x) and anti-hæmolytic changes
in the goat (No. 6) serum during and after series interposed injections with dog
cells. Arrow-heads indicate injections.

curves follow the same direction after the first series of antigen
administrations, and then turn in opposite directions during and after
the second series of injections.

At first sight the curves seem to suggest a close correlation between
the hæmolytic strength of the serum and the anti-hæmolytic potency

the immune serum a special factor or condition that antagonizes, neutralizes, or makes conditions unfavourable for, the anti-lytic action of cholesterol. This latter assumption seems to be supported by our experiments to be described below.

When a graded quantity of cholesterol (0·075–1·5 mg.) was added to a hæmolytic system containing the same amount of goat's immune lytic serum that caused 50–100 per cent. hæmolysis, it was observed that the increased inhibition of hæmolysis by increasing cholesterol concentration occurred only in the serum secured during the initial period of immunization, but failed to appear in the serum obtained
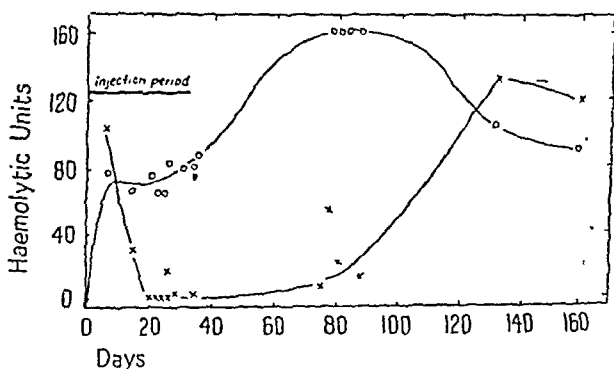
Fig. 2.—Graphs showing the hæmolytic potency (o-o-o) of the serum and the anti-hæmolytic potency (x-x-x) of the cholesterol in a goat (No. 1) during and after regular sustained injections with dog cells. The injections were made at 2 or 3 days intervals.

during the later stages of the development. This is shown by the graphs in fig. 3. Curve (a) is plotted from the data of the test made on a goat shortly after three injections with dog cells. It descends steeply towards the base-line, indicating a rapidly increasing inhibition of lysis by increasing cholesterol concentration. In this case hæmolysis was completely prevented even with a relatively low cholesterol content in the system. On the other hand, in the immune serum prepared from the same goat at a much later date, i.e. after prolonged repeated antigen administration, the hæmolysis-cholesterol concentration curve (b), (c), (d), (e), (f), (g) tends to flatten out or even slightly elevate with the increase of cholesterol concentration. It should be noted that the hæmolytic potency of the serum at this time was no stronger than that when the first test was made. However, about two months after the last injection, the serum, though still powerful in lysing dog cells, was no longer resistant to the anti-hæmolytic action of cholesterol. Thus the hæmolysis-cholesterol concentration curve (fig. 3, h and i) at this time resembles that obtained during the initial immunization.

Similar results were obtained in experiments on 2 goats with series interposed injections of dog cells. Fig. 4 shows graphically the changes

reduces or abolishes the cholesterol action, and in some cases may reverse it to a reinforcing action on hæmolysis.

DISCUSSION.

The anti-cholesterol property of the immune serum is very specific in that its manifestation requires the presence of the antigenic cell and specific complement. Thus, for instance, if the dog cell is replaced by the guinea-pig's in the immune rabbit serum and cholesterol system, the hæmolysis of the latter cell is reduced or prevented. This is illustrated by the data in Table I, where one sees that high concentra-

TABLE I.—COMPARISON OF CHOLESTEROL EFFECT ON THE IMMUNE RABBIT SERUM IN LYSING ANTIGENIC (DOG) AND NON-ANTIGENIC (GUINEA-PIG) CELLS.

Immune serum, ml.	Cells lysed.	Cholesterol (mg.) added to each 1·5 ml. system.			
		0.	0·075.	0·75.	1·5.
		Percentage hæmolysis.			
0·113	Dog	60	40	85	99
0·300	Guinea-pig	75	65	70	48

tions of cholesterol reinforce hæmolysis of dog cells but inhibit that of guinea-pig cells. In like manner, if the complement of the immune rabbit serum is substituted by that of normal guinea-pig's, cholesterol inhibition is again evident. As disclosed in Table II, no cholesterol

TABLE II.—COMPARISON OF CHOLESTEROL EFFECT ON TWO TYPES OF COMPLEMENT (ONE FROM IMMUNIZED RABBIT AND THE OTHER FROM NORMAL GUINEA-PIG).

Anti-dog sensitizing serum, ml.	Complementary serum, ml.	Cholesterol (mg.) added to each 1·5 ml. system.			
		0.	0·075.	0·75.	1·5.
		Percentage hæmolysis.			
0·15 (Rabbit)	0·075 (Rabbit)	60	60	65	65
0·15 (Rabbit)	0·015 (Guinea-pig)	58	50	40	34

inhibition was observed when heated immune rabbit serum was allowed to act with rabbit complement against dog cell, but this did not happen with guinea-pig complement. We have also carried out similar experiments with immune dog serum and obtained practically the same

of the cholesterol. But careful examinations reveal that the cholesterol activity suffers a great reduction at the time when the hæmolytic potency is still advancing, and recovers before the decline of the lytic power of the serum.

3. *Experiments with Dogs.*—That hæmolysis of rabbit cells induced by normal dog serum can be prevented by cholesterol has been demonstrated in our previous investigation [Lee and Tsai, 1941]. What would happen if washed rabbit cells are injected into the dog? Does the latter also acquire a special factor against cholesterol action? To settle this problem, we have performed experiments on 5 dogs. After
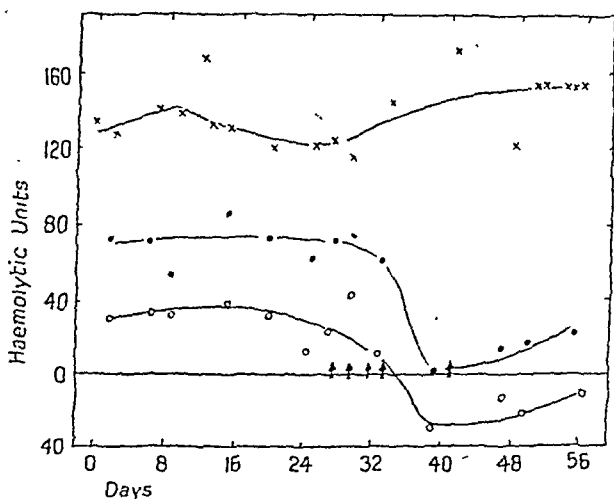
Fig. 5.—Graphs showing the hæmolytic and anti-hæmolytic changes in the dog (No. 1) serum before and after repeated injections with rabbit cells, x-x-x, hæmolytic curve; •····, anti-hæmolytic curve obtained with 0·375 mg. cholesterol in the 1·5 ml. system; o-o-o, anti-hæmolytic curve with 0·075 mg. cholesterol in another corresponding system. Arrow-heads signify injections.

a prolonged control observation the animals were injected with washed rabbit cells four or five times at intervals of 2–10 days. The results are consistent and conclusive in showing that the serum, the hæmolytic strength of which increased only slightly, invariably became refractory to cholesterol action after the third or fourth injection of the antigen. Fig. 5 shows the typical course of hæmolytic and anti-hæmolytic changes. In a number of tests we found that cholesterol may exert a reinforcing action on hæmolysis of the rabbit cell by the immune dog serum. This occurred more frequently and strikingly at low than at high concentrations of cholesterol (fig. 5), which is the opposite to what was observed in rabbit and goat immune serum. The reason for this discrepancy is not known. At any rate, it leaves no room for doubt that the serum has developed, by immunization, a condition that

from the attack of lytic agents, it is conceivable that it may do the same to foreign cells introduced from without. If that is so, then the anti-cholesterol property may play an important rôle in immunity, because it may prevent the plasma cholesterol from protecting the invading cells and thereby leave them free to be dealt with by the lytic agents in the body. Although this hypothesis is, of course, purely speculative, it opens up for research many problems the solution of which may throw light on the long-debated questions regarding the relation of cholesterol to immunity.

SUMMARY.

1. A specific property that antagonizes the anti-hæmolytic action of cholesterol, or may even reverse it to an accelerating one on hæmolysis, has been demonstrated in the sera of rabbits, goats, and dogs immunized with dog cells, dog cells and rabbit cells respectively.

2. This property is always associated with the acquired sensitizers, but its development does not run exactly parallel with them.

3. The specificity of the anti-cholesterol property is demonstrated by the fact that it acts only in the presence of specific antigen and complement.

4. It is not related to the agglutinating property or process of the immune serum.

REFERENCES.

LEE, J. S., and TSAI, C. (1941).   *Proc. Chinese Physiol. Soc. Chengtu*, Br. **1**, 15.
LEE, J. S., and TSAI, C. (1942).   *Ibid.* **1**, 49.
LEE, J. S., and TSAI, C. (1943).   *Ibid.* **1**, 111.

results. Both normal and immune dog sera are able to lyse human and guinea-pig cells, and the hæmolysis of the latter can be prevented by cholesterol in proper concentration. If now the rabbit cell (antigen) is replaced by human or guinea-pig's, the immune dog serum no longer resists the anti-lytic action of cholesterol. Similarly, if the guinea-pig's complement is employed in place of dog's, the inhibitory behaviour of cholesterol is again manifested. It is clear then that the anti-cholesterol property of the immune serum is almost as specific as the sensitizer. This statement raises the question as to whether this property is characteristic of the new sensitizer. No doubt they are closely associated with each other, but whether the anti-cholesterol property can be independent of the new sensitizer requires further experiments for elucidation.

The most interesting fact demonstrated in the present experiment is the reaction of dog serum. Although the lytic action of normal dog serum toward rabbit cells can be easily inhibited by cholesterol, this is no longer so, or the action may even be reversed to a reinforcing effect on hæmolysis after a few injections of rabbit cells. Whether the accelerating phenomenon is due to the same property that prevents the cholesterol inhibition remains to be studied. At any rate, the development of this property seems to be a general body reaction in response to the introduction of the antigen, irrespective of the presence or absence of the natural hæmolytic sensitizer.

The development of this property in the serum is not exactly parallel with the rate of formation of the new sensitizers, because in the goat it becomes manifest only after prolonged immunization, and wanes before the disappearance of the hæmolytic element, while in the dog this factor is exceedingly strong despite the small increase of the hæmolytic strength. Although our data provide no justification for drawing a conclusion regarding the precise relationship between this property and the sensitizer, we believe, on the basis of the above findings, that the development of this property is not due to the excessive accumulation of sensitizers.

It may be remarked that the immune sera of all the animals used always develop an agglutinating reaction. Does the agglutinin or the agglutinating process affect or interfere with the action of cholesterol? Our experience shows this not to be so, because the agglutinin titre is usually not parallel with the onset of the change of the serum towards the cholesterol action. For instance, in many cases the serum has developed this latter factor long before it exhibits any detectable agglutinating property.

What physiological significance does the acquisition of this anti-cholesterol property of the serum have? It is rather premature to make a fruitful conjecture at present. But, if we assume that the normal function of the plasma cholesterol is to safeguard the red cells

THE PROTECTIVE ACTION OF THE SERUM AGAINST NATURAL HÆMOLYSIN.[1]

By Jui-Shuan Lee and Chiao Tsai. From the Department of Physiology, College of Medicine, National Central University, Chengtu, China.

That dog serum is hæmolytic to washed rabbit red cells is well known. In our previous communication [Lee and Tsai, 1942] we have reported that the activity of this normal hæmolytic serum (natural hæmolysin) towards rabbit cells can be inhibited by cholesterol and lecithin. In the present experiment similar action was observed with rabbit serum. The present paper deals with a series of experiments attempted to elucidate the mechanism of this inhibitory action of the serum.

METHODS.

The methods used in the present study were practically the same as those described in our previous publication [Lee and Tsai, 1942]. Dog serum was prepared from blood withdrawn from the external jugular vein. Rabbit blood was obtained by bleeding the animal from the carotid artery under general ether anæsthesia, or in some cases under local narcosis with cocaine. All the samples were left at room temperature for 30–60 minutes and the serum was separated off from the clot by centrifugation. The hæmolytic system consisted of 0·03 ml. of 50 per cent. washed rabbit cells, a variable amount of hæmolytic and anti-hæmolytic sera and a variable amount of isotonic buffered saline, making up exactly to a final volume of 1·5 ml. The percentage of hæmolysis was determined colorimetrically after the system was incubated at 37° C. for 30 minutes.

In some experiments the rabbit and dog sera were mixed together and incubated at 37° C. for 30 minutes before adding to the system containing cells. In some other experiments the anti-hæmolytic rabbit serum was heated at 55°–56° C. for half an hour before use. In still another series of experiments sensitized rabbit cells were employed. They were prepared by mixing the washed rabbit cells with 10 volumes of heated dog serum (HDS), which was then incubated at 37° C. for two hours. At the end of incubation the mixture was centrifuged, the serum removed, and the cells washed thrice with normal saline. The

variations are the main characteristics of the interaction of FDS and RC with graded quantities of FRS found in practically all our experiments. Further analysis reveals that they are conditioned by a number of relevant factors, which we shall describe separately in the following sections.

2. *Cholesterol not the Sole Agent involved in this Protective Mechanism.*

That cholesterol is anti-complementary toward the normal hæmolytic serum has been clearly demonstrated in our experiments reported in the preceding paper. But that it is not the sole factor in the protective mechanism of FRS against the hæmolytic action of FDS on RC is shown by the following experiment. When cholesterol was employed as the anti-hæmolytic agent in place of FRS, the reduction of RC hæmolysis induced by FDS seemed to vary with, though not exactly proportional to, the quantity of cholesterol. As shown in fig. 1, c, the rate of hæmolysis decreases with the concentration of cholesterol in the system, at first very rapidly and then followed by a negative acceleration. It is not so if cholesterol is replaced by FRS. This may be taken to mean that the anti-hæmolytic action of FRS is different in nature from that of cholesterol alone. We may therefore postulate that cholesterol is one of the factors in FRS that exerts the protective action on the cells against FDS, but it cannot be the only one. In the following series of experiments it will be shown that sensitization of rabbit cell by dog serum, as well as the presence of an anti-sensitizing factor, also play an important rôle in this complex protective mechanism of the serum.

3. *The Effect of Sensitization.*

We have already mentioned that washed rabbit cell can be sensitized by heated dog serum and subsequently hæmolysed by its own serum. When FDS and FRS were added together to the sensitized rabbit-cell (SRC) suspension, the extent of hæmolysis was much greater than when FDS was used alone (compare fig. 2, a with 2, b). Here the protective action of FRS is entirely masked. We may assume provisionally that, in the case of initially unsensitized cells, the cells may become gradually sensitized after being mixed and incubated with FDS and that after partial sensitization the FRS may act hæmolytically toward rabbit cells if present in sufficient quantity. In other words, in such a system the FRS may exert two opposite actions: an anti-hæmolytic one, largely due to cholesterol and allied substances, and a hæmolytic one, due to its complement acting upon the partially sensitized cells. The latter process can, however, take place only after an interval of time necessary for sensitization. The time factor is eliminated when washed RC has been previously sensitized by HDS. In this case the rabbit complement can at once attack its own cell upon their being brought

washed cells were diluted with buffered saline up to 50 per cent. suspension. The cells so treated may be hæmolysed by the serum of the same animal and are said to be sensitized; *i.e.* the rabbit cells are now endowed with dog amboceptor and thus rendered sensitive to the hæmolytic action of the complement of its own serum.

RESULTS.

1. *The Complex Nature of the Protective Action of the Serum.*

When fresh rabbit serum (FRS) was added together with fresh dog serum (FDS) to the washed rabbit cell suspension (RC), the hæmolytic
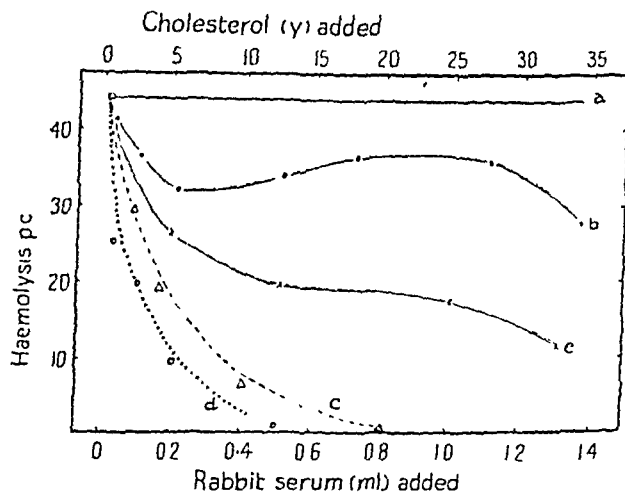
FIG. 1.—Showing the effect of hæmolytic normal dog serum on the initially unsensitized rabbit cell. *a*, FDS (0·075 ml.) + RC; *b*, FDS + FRS + RC, simultaneously mixed; *c*, FDS + cholesterol, incubated, then RC added; *d*, FDS + HRS, incubated, then RC added; *e*, FDS + FRS, incubated, then RC added.

activity of the dog serum was reduced, indicating the protective action of FRS for RC. However, the anti-hæmolytic activity of FRS was not proportional to its quantity in the system. This is illustrated by the results of a typical experiment graphically represented in fig. 1. When 0·075 ml. FDS alone was employed in the system, the magnitude of hæmolysis of RC was approximately 45 per cent. (curve *a*). On the other hand, when FRS was introduced into the system in addition to FDS, the extent of hæmolysis became lessened (curve *b*). The curve in this case appears rather complex. In a number of experiments we always observed the least hæmolysis with low concentrations of FRS. With higher concentrations, the degree of hæmolysis was not further reduced; as a matter of fact, in a few cases it approached almost the same level as, or even far beyond, the line when FDS was allowed to act alone on RC. Nevertheless, a further increase of FRS tended in all cases to bring down the percentage of hæmolysis again. These

again as the amount of FRS is further increased (fig. 1, b). This phenomenon cannot be interpreted by the action of the factors already enumerated, but must be conditioned by some other factor existing in the FRS that causes a partial inhibition of the sensitization of RC induced by FDS. The presence of the anti-sensitizing factor in rabbit serum is revealed when an ample quantity of rabbit serum is present in the system during the process of sensitization. For the sake of brevity we can only present a typical example as follows: A series of samples containing 0·03 ml. RC suspension, 0·3 ml. HDS, and a variable amount of FRS were incubated at 37° C. for 30 minutes. The cells in the system were then washed three times with buffered saline so as to free them completely from the adhesion of the sera. After proper dilution the degree of sensitization was tested by reintroducing 0·2 ml. of FRS or FDS into the cell suspension and determining the percentage of hæmolysis. The results of this experiment are given in Table I. It is seen that the sensitization of RC by FDS is lessened or prevented by FRS, depending to a large extent upon the relative amount of the latter introduced into the sensitizing system.

We have also tested heated rabbit serum (56° C. for 30 minutes) and found that it possesses an equal or even slightly stronger inhibitory

TABLE I.—THE INHIBITORY ACTION OF FRS ON THE SENSITIZATION OF RC BY HDS.

Sample No.	1.	2.	3.	4.	5.	6.	7.
For sensitization { HDS ml. . .	0·3	0·3	0·3	0·3	0·3	0·3	0·3
FRS ml. . .	0	0·01	0·02	0·05	0·1	0·5	1·0
Hæmolysis per cent. by 0·2 ml. FRS after sensitization.	11	12	5	5	2	0	0

TABLE II.—THE INHIBITORY ACTION OF FRS AND HRS ON THE SENSITIZATION OF RC BY HDS.

FRS or HRS ml. added during sensitization.	Without previous sensitization.		With previous sensitization.			
	0.	0.	0·075.	0·15.	0·75.	1·35.
Hæmolysis per cent. by FDS after sensitization. I. { (a) With previous incubation.	20	36	34	33	30	26
(b) Without .	20	36	37	34	30	26
II. { (a) With previous incubation.	20	36	32	31	25	21
(b) Without .	20	36	32	..	26	21

I, using FRS as anti-sensitizing agent.
II, using HRS as anti-sensitizing agent.

together, causing more marked hæmolysis after a short period of *incubation*. On the other hand, when FDS and FRS are allowed to act simultaneously upon the initially unsensitized rabbit cells, the immediate reaction is, of course, an inhibition on account of the anti-complementary action of FRS on the hæmolytic FDS. This is followed sooner or later by a diminution of inhibition when RC is sufficiently sensitized by dog serum, that is when the hæmolytic action of rabbit serum overpowers the anti-hæmolytic strength of cholesterol and allied
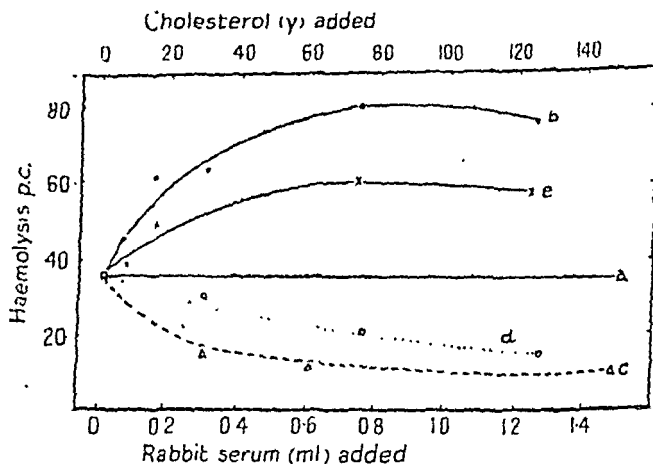
FIG. 2.—*Showing the effect of hæmolytic normal dog serum on previously sensitized cell. a,* FDS (0·113 ml.) +SRC; *b,* FDS +FRS +SRC, simultaneously mixed; *c,* FDS +cholesterol, incubated, then SRC added; *d,* FDS +HRS, incubated, then SRC added; *e,* FDS +FRS, incubated, then SRC added.

substances contained in it. This explains the rise of the hæmolysis curve with a certain increased quantity of FRS (fig. 1, *b*).

It is pertinent to remark that the effect of FRS is quite different from that of HRS on the sensitization of RC by FDS. Careful examination of the graphic records in figs. 1 and 2 (compare fig. 1, *c* with 1, *d* for initially unsensitized cell, and fig. 2, *c* with 2, *d* for sensitized cell) reveals the fact that the anti-hæmolytic behaviour of HRS is very similar to that of cholesterol. Since HRS contains no complement, it can no longer hæmolyse RC even after the latter has been sensitized by dog serum. This would probably explain the absence of rise of the hæmolysis curve with the increased quantity of HRS added to the system. In other words, the anti-hæmolytic action of HRS must be largely due to cholesterol and allied substances.

4. *The Presence of an Anti-sensitizing Factor and its Influence.*

It is of prime importance to note that the hæmolysis curve obtained from the FDS and RC system in the presence of FRS tends to decline

CHANGES IN THE CIRCULATING BLOOD IN MAN FOLLOWING BLOOD LOSS OR TRANSFUSION OF CONCENTRATED RED CELLS.[1] By M. DYSON, G. PLAUT, and JANET VAUGHAN.

THE objects of the present paper are (i) to describe observations which show that extremely rapid changes may take place in the distribution of fluid between the tissues and the circulating blood in man, (ii) to emphasise the need to recognise that such changes may occur when assessing the effect of either the removal or administration of blood.

The experiments on blood loss were undertaken as part of an investigation into the factors that might be responsible for vasovagal attacks or "faints" in blood donors. The observations on the effect of transfusion of concentrated red cells were begun in order to check the dye method of estimating blood volume against the concentrated corpuscle differential agglutination method [McMichael *et al.*, 1943].

EXPERIMENTAL PROCEDURE.

Observations were made on 8 healthy subjects before removal of 540 c.c. of blood and for a two-hour period after bleeding, and on 8 severely anæmic individuals before and after a transfusion of approximately 450 c.c. of concentrated red cells [MacQuaide and Mollison, 1940].

In the case of the healthy subjects the following procedure was adopted. Having had their ordinary breakfast they reached the bleeding depot at 9 o'clock and lay down for half an hour covered with a blanket. They were given sufficient hot-water bottles to keep them comfortably warm. At 9.30 a sample of blood was withdrawn from a vein in the anticubital fossa without congestion. Evans blue dye was then injected into the vein of the other arm as described below. Further samples of approximately 15 c.c. each were then withdrawn at half-hourly intervals for 1½ hours, with the exception of Case 8 when the withdrawal took place after 45 minutes. At the end of this time 540 c.c. of blood were removed by the usual depot procedure. In order to calculate the quantity of cells and plasma removed in 540 c.c. of

[1] Report to the Medical Research Council from the N.W. London Blood Supply Depot.

power on the sensitization process, *indicating the thermostability of the anti-sensitizing factor.* Table II records the results of a typical experiment using FDS (0·075 ml. in 1·5 ml. containing 1 per cent. sensitized *cell suspension*) as the hæmolysing agent. In this experiment the cells in saline suspension (1 per cent.) were sensitized by HDS (0·075 ml. in 1·5 ml.). It is noted that when sufficient FRS is added to the system, the extent of hæmolysis is comparable with that without previous sensitization; *i.e.* the process of sensitization in this case is completely inhibited. It should also be remarked that *previous incubation of FDS with FRS (at 37° C. for 60 minutes) does not heighten the process of sensitization.* The absence of such an influence may be taken to mean that the inhibitory action of FRS on the sensitization of RC by HDS can occur only in the presence of cells.

When FDS and FRS are mixed and incubated before adding to RC suspension, the extent of hæmolysis is relatively less (figs. 1, *e* and 2, *e*) than when they are mixed with the cells without previous incubation (figs. 1, *b* and 2, *b*). According to the observations described in the preceding paragraphs, one cannot escape the conclusion that the difference between these two cases is due to the partial inactivation of complement by the anti-complementary mechanism of *both rabbit and dog sera.*

Summary and Conclusion.

1. Rabbit serum exhibits *an inhibitory behaviour towards the hæmolytic action of dog serum on rabbit cells.* The protective power of rabbit serum increases after the destruction of its complement by heating.

2. Rabbit serum may hæmolyse its own cells after the latter have been sensitized by heated dog serum.

3. Rabbit serum, either fresh or heated, is able to reduce or abolish the sensitizing action of dog serum, presumably *due to the existence of a thermostable anti-sensitizing factor in the rabbit serum.*

4. It is therefore concluded that the protective action afforded by rabbit serum against the hæmolytic action of dog serum on rabbit cells may be attributed to two factors: the anti-complementary factor, which, according to our previous investigation, is largely due to cholesterol and allied substances, and an anti-sensitizing factor, which inhibits the sensitization of rabbit cells by dog serum, and thus prevents the rabbit serum from becoming hæmolytic to its own cells. The exact nature of the anti-sensitizing factor is unknown.

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CHANGES IN THE CIRCULATING BLOOD IN MAN FOLLOWING BLOOD LOSS OR TRANSFUSION OF CONCENTRATED RED CELLS.[1] By M. Dyson, G. Plaut, and Janet Vaughan.

The objects of the present paper are (i) to describe observations which show that extremely rapid changes may take place in the distribution of fluid between the tissues and the circulating blood in man, (ii) to emphasise the need to recognise that such changes may occur when assessing the effect of either the removal or administration of blood.

The experiments on blood loss were undertaken as part of an investigation into the factors that might be responsible for vasovagal attacks or "faints" in blood donors. The observations on the effect of transfusion of concentrated red cells were begun in order to check the dye method of estimating blood volume against the concentrated corpuscle differential agglutination method [McMichael *et al.*, 1943].

EXPERIMENTAL PROCEDURE.

Observations were made on 8 healthy subjects before removal of 540 c.c. of blood and for a two-hour period after bleeding, and on 8 severely anæmic individuals before and after a transfusion of approximately 450 c.c. of concentrated red cells [MacQuaide and Mollison, 1940].

In the case of the healthy subjects the following procedure was adopted. Having had their ordinary breakfast they reached the bleeding depot at 9 o'clock and lay down for half an hour covered with a blanket. They were given sufficient hot-water bottles to keep them comfortably warm. At 9.30 a sample of blood was withdrawn from a vein in the antecubital fossa without congestion. Evans blue dye was then injected into the vein of the other arm as described below. Further samples of approximately 15 c.c. each were then withdrawn at half-hourly intervals for 1½ hours, with the exception of Case 8 when the withdrawal took place after 45 minutes. At the end of this time 540 c.c. of blood were removed by the usual depot procedure. In order to calculate the quantity of cells and plasma removed in 540 c.c. of

[1] Report to the Medical Research Council from the N.W. London Blood Supply Depot.

blood when a pressure of 80 mm. Hg was maintained above the vein. *a sample of blood was taken from the blood withdrawal apparatus at* the beginning of bleeding and another at the end of bleeding. Hæmatocrit estimations were made on both samples and the mean of the two readings was taken to represent the hæmatocrit value of the 540 c.c. of blood withdrawn: from this figure the total quantity of red cells and plasma removed was calculated. Further samples were collected immediately after bleeding from the opposite arm and at quarter-hourly intervals for one hour and a final sample two hours after bleeding, the subject remaining at rest. One cup of tea was given to all subjects after the removal *of 540 c.c. of blood, in order to make conditions similar to* those of the ordinary blood donor.

In the case of anæmic patients receiving transfusions the initial plasma volume was determined by the same method as that described later for normal subjects. The patients were then given a transfusion of 450–520 c.c. of concentrated red cells, and further samples of blood for estimation of plasma volume were withdrawn within a quarter of an hour of finishing the transfusion and two hours later. No attempt was made to standardise either the age or the concentration of the red cells given. *In subsequent experiments a standard concentrate only should* be used.

(i) *Hæmatological Investigations.*

The following observations were made upon each sample: red cell counts, hæmoglobin estimations, cell volume and mean corpuscular volume, upon heparinised venous blood by methods previously described [Price Jones, Vaughan, and Goddard, 1935]. All the red-cell counts and hæmoglobin estimations were made by one observer (J.V.). Differences greater than 200,000 per cm. in the red-cell count are probably significant. Hæmoglobin is read to an accuracy of ± 2 per cent. on the scale. The estimation of mean corpuscular volume is more open to error as it is calculated from the red-cell count and the packed cell volume. Brown *et al.* [1942] have estimated that changes in mean corpuscular volume are not significant unless they exceed 6·4 per cent. The possibility of double error applies also to mean corpuscular hæmoglobin and mean corpuscular hæmoglobin concentration. On one individual 11 estimations of mean corpuscular hæmoglobin throughout the day varied by as much as 1·67 $\gamma\gamma$ and 12 estimations of mean corpuscular hæmoglobin concentration per cent. varied by as much as 1·91 per cent. Changes in any of the above values that are maintained over a series of observations are clearly more likely to be significant than changes noted at a single reading. The latter might well be due to personal error when the large number of observations made during the experimental period is remembered.

(ii) *Plasma Proteins.*

In the early cases plasma or serum protein was estimated by the method of King *et al.* [1937, 1942]. Differences greater than 0·4 g. are considered significant. In the later cases the Micro-Kjeldahl technique was employed—differences greater than 0·25 g. are then considered significant [Dyson and Plaut, 1943].

(iii) *Plasma Volume.*

The plasma volume was determined by Harington, Pochin, and Squire's [1940] modification of the method of Gibson and Evans [1939]. Evans blue dye, 12 mg. dissolved in 5 c.c. of saline, was injected from a calibrated syringe into the median cubital vein, the syringe being washed out with blood three times. Samples for the determination of the disappearance curve for the dye were withdrawn from the vein on the other arm. Estimations were made on serum samples rather than plasma since hæmolysis does not occur so readily in serum. The concentration of dye when extracted was estimated in a Hellige colorimeter in a dark room. Ten different estimations made on one sample showed a variation in plasma volume of 3·9 per cent., the biggest actual difference observed being 116 c.c. on a total plasma volume of 3042 c.c. This includes the errors in the colorimetric reading and those inherent in the different procedures involved in sampling and extracting the dye. The initial plasma volume was obtained by extrapolating back from the first three points of the disappearance curve of the dye, *i.e.* from the concentration of dye in samples taken at $\frac{1}{2}$ hour, 1 hour, and $1\frac{1}{2}$ hours after injection. Subsequent calculations of plasma volume were made by extrapolation back parallel to this initial disappearance curve from any point obtained for concentration of dye, a modification of the method suggested by Gibson and Evans [1939]. In order to check how far this method is likely to be satisfactory in our hands and using the dye sample available to us, dye was given in the usual way to a normal subject, and a disappearance curve plotted over this period, the plasma volume for each point on the curve being calculated by the method described above. A further injection of dye was then given at the end of 24 hours and a fresh disappearance curve plotted from points obtained $\frac{1}{2}$ hour, 1 hour, and $1\frac{1}{2}$ hours after injection of the dye (Table I). The reasons for the irregularity of the curve observed are discussed below. Estimations of plasma volume calculated from both the first disappearance curve at the end of 24 hours and the second disappearance curve differed by only 58 c.c., which is considerably less than the difference observed on ten estimations on the same sample. The method of extrapolation as used, therefore, appears satisfactory in our hands. The results obtained by our method of calculation gave

TABLE I.—PLASMA VOLUME AND HÆMATOLOGICAL VALUES IN A NORMAL AMBULATORY SUBJECT.

Time.	Plasma volume, c.c.	Cell volume, c.c.	Red cells, mills. per c.c.	Hæmo-globin, per cent.	Packed cell volume, c.c.	M.C.V., cµ.	M.C.H., γγ.	M.C.H.C., per cent.	Plasma protein (King's method).
10.12 a.m.			4430	84	39	88·02	26·16	29·72	7·5
10.22	12 mg.*								
10.52	3133	2024	4480	84	39	85·15	25·31	29·72	7·5
11.22			4410	86	39·5	89·58	26·92	30·05	7·6
11.52			4400	86	39·5	89·79	26·98	30·05	7·9
4.22 p.m.	3192	2218	4520	87	40·5	89·60	26·55	29·63	7·6
7.08	3519	2396	4490	86	40	89·08	26·44	29·68	7·8
10.15	3750	2553	4430	84	40	90·30	26·16	28·97	7·8
9.54 a.m.	3429	2358	4500	83	40	89·46	25·45	28·45	7·6
10.00	12 mg. dye given								
10.30	3371	2319	4480	84	39·5	88·17	25·87	29·34	7·7
11.00			4500	84			25·75		7·4
11.31			4410	86			26·92		7·2

* Dye given.

comparable though rarely precisely identical results with those obtained by the use of the formula suggested by Gibson and Evans [1939]. In order to determine whether fluctuations in plasma volume may occur spontaneously in a resting subject, seven estimations were made on one individual during a period of 13 hours (Table II). The maximum

TABLE II.—PLASMA VOLUME AND HÆMATOLOGICAL VALUES IN A NORMAL RESTING SUBJECT.

Time.	Plasma volume, c.c.	Cell volume, c.c.	Hæmo-globin, per cent.	Packed cell volume, c.c.	M.C.H.C., per cent.	Plasma protein (King's method).
9·38 a.m.			82	37	30·57 ·	7·3
9·41	12 mg.*	—				
10.10	2857	1678	82	37	30·57	7·4
10.41			82	37	30·57	7·4
11.11			82	37	30·57	7·4
12.30 p.m.	2837	1739	83	38	30·14	7·3
2.31	2797	1678	84	37·5	30·91	7·2
4.29	2824	1658	81	37	30·16	7·2
6.29	2913	1747	82	37·5	30·16	7·3
8.36	2877	1764	84	37·5	30·90	7·3
10.28	2850	1784	82	38	29·77	7·2
6.46 a.m.			82	39	29·0	7·2
9.48			82	39	29·0	

* Dye given.

difference observed was 116 c.c. again within the limits of the error of the method. It is, however, essential if small differences are to be accepted to keep the subject at complete rest. In a series of observations made on the same subject when ambulatory, differences in plasma volume as big as 627 c.c. were observed between different samples (Table I). In all the experiments recorded here, therefore, the subjects were maintained at complete rest, lying down during observation. In order to obtain figures for plasma volume after blood withdrawal, it appeared necessary to allow for the dye removed in the 540 c.c. of blood. This was calculated in the following way. The total amount of blood removed was measured, the relative quantities of plasma and cells in the removed blood were estimated as already described from the hæmatocrit readings of the samples taken at the beginning and end of bleeding. The concentration of dye in the plasma removed was also estimated, and knowing the total volume of this plasma it was then possible to calculate the amount of dye removed. In any subsequent calculations of plasma volume allowance was made for this removed dye. The points obtained for dye concentration after withdrawal were extrapolated back parallel to the original disappearance curve of the dye. From the reading so obtained, knowing the amount of dye left in the circulation, the plasma volume was calculated in the usual way. In one instance the above corrections were also made for withdrawal of dye with each sample: the differences were so small from reading to reading that it was felt justifiable to ignore such corrections. It may be objected that after removal of some dye the disappearance curve of what remains may be altered, and further that the curve may be affected by any alteration in plasma volume that occurs. There is at present no means of controlling these possible sources of error. The effect is, however, likely to be small in comparison to the differences observed.

The figures for plasma volume, cell volume, and blood volume that might be expected after removal of 540 c.c. of blood which are shown in the charts as calculated volume were obtained by subtracting the volume removed from the volume observed before bleeding. In the same way the figures for plasma volume, cell volume, and blood volume that might be expected after transfusion of red cells, expressed as calculated volume, were obtained by addition of the volume given to the volume observed before bleeding.

<center>RESULTS.</center>

<center>*Withdrawal of 540 c.c. of Blood.*</center>

The results of removing 540 c.c. of blood were variable. Three types of response were obtained:

Type I: No Dilution.—A woman of 36 (K. G.) showed an approxi-

mately constant red-cell count and hæmoglobin before and after bleeding. The plasma volume fell to the calculated level and remained there for the two-hour period (see fig. 1 and Table III).
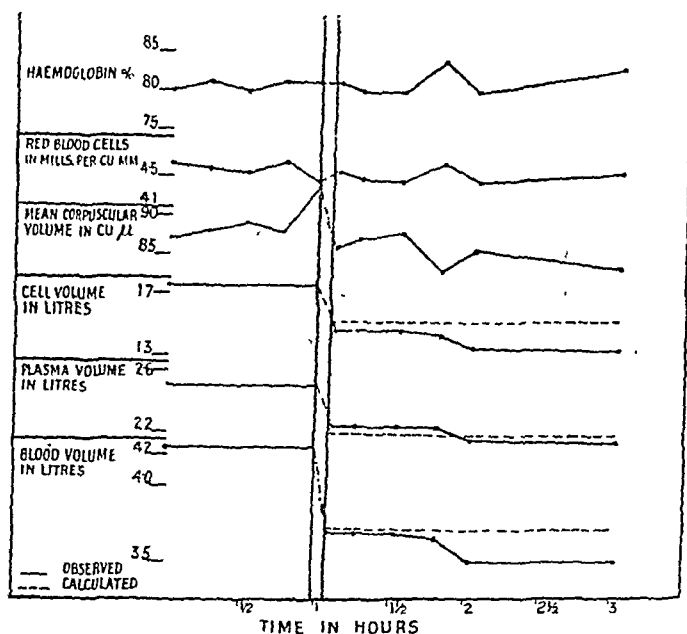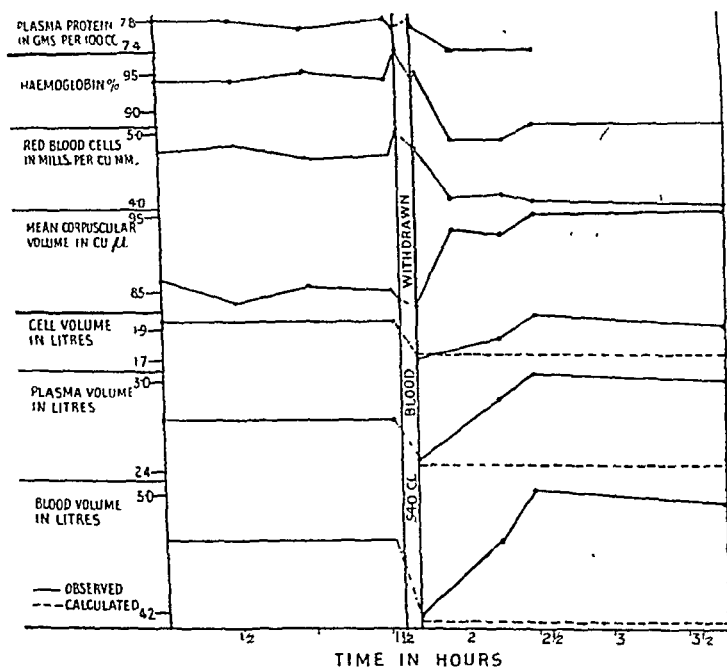

Fig. 1.—Effect of removal of 540 c.c. of blood on hæmoglobin, red cells, mean corpuscular volume, cell volume, plasma volume, and blood volume in a normal individual. No dilution.

TABLE III.—MAXIMUM EFFECT OBSERVED IN TWO-HOUR PERIOD FOLLOWING REMOVAL OF 540 C.C. OF BLOOD.

Case.	Plasma volume, c.c.	Cell volume, c.c.	Hæmo-globin, per cent.	R.B.C., thousands per cm.	M.C.V.. cµ.	M.C.H., γγ.	M.C.H.C., per cent.	Proteins, g. per cent.	Method.
1. M. E.	+612	+263	− 8	−600	+10	+1·98	−0·63	−0·4	King
2. G. P.	+342	+163	− 8	−600	+ 8	+0·68	−1·46	no change	King
3. N. M.	+355	+ 30	−10	−800	+13	+1·79	−1·79	−0·4	Micro-Kjeldahl
4. H. J.	+313	+291	− 8	−700	+14	+1·83	−2·47	−0·14	Micro-Kjeldahl
5. L. G.	+305	+260	− 7	−700	+10 {	−1·23 +1·3	−2·5	−0·5	King
6. H.	+201	+118	− 8	−300	no change	−1·08	−0·89	−0·6	King
7. T.	+200	+105	− 6	−600	+11 {	−0·51 +1·66	−1·35	no change	King
8. K. G.	+ 45	−174	no change	no change	− 5	+0·90	+2·58	..	

Type II: Overdilution.—A woman of 48 (M. E.) showed considerable overdilution as evidenced by a plasma volume greater than she had before bleeding (Table III and fig. 2). Her hæmoglobin fell from 94 per cent. to 86 per cent. Her plasma volume at the peak was 620 c.c. above the calculated figure, 218 c.c. above the initial value. In addition her total cell volume was increased by 263 c.c. above the

Fɪɢ. 2.—Effect of removal of 540 c.c. of blood on hæmoglobin, red cells, mean corpuscular volume, cell volume, plasma volume, blood volume, and serum protein in a normal individual. Overdilution.

calculated volume and 39 c.c. above the original volume due to an increase in mean corpuscular volume. In other words, approximately 883 c.c. of fluid had come into the circulation from the tissues.

Type III: Moderate Dilution.—The other six donors showed a varying increase in plasma volume above the calculated figure (Table III), the initial volume being restored within 2 hours in some cases. The time at which dilution occurred was variable. It appeared to have already started by the time the blood withdrawal was finished and reached a maximum at the end of 1 hour with one exception when a maximum was found at the end of 2 hours. In the others a slight decrease in plasma volume was noticed at the end of 2 hours. The increase in plasma volume was associated except in one instance with an appreciable and maintained increase in mean corpuscular volume.

There was an associated fall in hæmoglobin and red-cell count, which was also maintained through the period of observation.

In none of the subjects was a rise in serum protein noted, and in three instances there was an appreciable fall. In 6 out of the 8 cases the change observed in mean corpuscular hæmoglobin after bleeding was no greater than that observed in a normal individual throughout the day. In two instances it was slightly greater, being $1 \cdot 98 \, \gamma\gamma$ and $1 \cdot 83 \, \gamma\gamma$ respectively, the biggest variation observed in the control being $1 \cdot 67 \, \gamma\gamma$. It is doubtful whether the difference is significant, so that it may be concluded there was no appreciable change in mean corpuscular hæmoglobin. The mean corpuscular hæmoglobin concentration per cent. changed inversely to the mean corpuscular volume, though again with three exceptions the change was no greater than that seen in the control. The fact that there was no significant change in these values suggests that both the hæmoglobin and red-cell figures are correct.

TRANSFUSION OF CONCENTRATED RED CELLS.

In the eight anæmic individuals receiving transfusions of concentrated red cells the data are less complete, as it is not possible to perform venepunctures at will on extremely ill patients. In four patients there was an increase in plasma volume after transfusion above that calculated varying from 442 c.c. to 262 c.c. (Table IV and fig. 3). In three patients there was a decrease in plasma volume varying from 241 to 253 c.c.— in one patient the plasma volume was that calculated within 2 c.c. (Table IV and fig. 4). In those patients in whom both immediate and 2-hour samples were available the maximum increase had occurred at 2 hours, though some increase was seen immediately. In two instances increase in plasma volume was associated with a fall in plasma protein of 0·8 g. and 1·1 g. respectively. In two further instances an increase in plasma volume was associated with no change in plasma protein. In no case was a decrease in plasma volume associated with any marked change in plasma protein.

In Case 7 (Table IV and fig. 4) it may be noted that the total cell volume is considerably less than expected, though there is a rise in red cells and hæmoglobin. The cells used had been prepared from blood taken into an experimental diluent. Cells taken into this diluent show considerable swelling, which gives a high packed cell volume for the blood previous to administration. The calculated cell volume after transfusion is in fact based on this hæmatocrit reading. When such swollen cells are put into the circulation they come into contact with normal plasma and on theoretical grounds might be expected to lose fluid and therefore become smaller. That such loss of fluid occurred is shown by the fact that the mean corpuscular volume of the red cells is no greater after transfusion than it was before, though large cells

TABLE IV.—EFFECT OF TRANSFUSION OF 450–520 C.C. CONCENTRATED RED CELLS.

Case No.	Type of anæmia	Difference between observed and calculated plasma volume, c.c.		Difference between observed and calculated cell volume, c.c.		Difference between observed and calculated blood volume, c.c.		Observed change in M.C.V.		Observed change in Hb per cent.		Observed change in R.B.C. thousands per cm.		Observed change in serum proteins in g.		Method.	Amount given, c.c.
		Within 15 min.	After 2 hrs.	Within 15 min.	After 2 hrs.	Within 15 min.	After 2 hrs.	Within 15 min.	After 2 hrs.	Within 15 min.	After 2 hrs.	Within 15 min.	After 2 hrs.	Within 15 min.	After 2 hrs.		
1	Pernicious Anæmia	+442		+ 92		+534		–11·89		+14		+800		– 0·8		King	470
2	Anæmia Pregnancy	+264	+ 360	+158	+204	+422	+564			+11	+ 9	+530	+520	no change	no change	Micro-Kjeldahl	450
3	Anæmia? Carcinoma	+339	+ 57	+121	– 56	+460	+ 1	– 2·47	–8·36	+13	+ 13	+680	+660	no change		King	520
4	Iron deficiency Anæmia	+262	+ 228	+ 67	+ 21	+339	+249			+14	+ 14	?	+710	– 1·1	– 1·1	King	510
5	Iron deficiency Anæmia	+		+117		+119				+12	+ 12	+720	+780	– 0·44		Micro-Kjeldahl	470
6	Pernicious Anæmia *	–174	– 409	+113		– 61		–25·73		+18		+910		– 0·28		Micro-Kjeldahl	468
7	Anæmia? Hæmolytic	– 62	– 253	–127	–217	–189	–470	– 2·55	–3·47	+ 7	+ 7	+410	+380	– 0·22	– 0·23	Micro-Kjeldahl	480
8	Pernicious Anæmia	–211		– 36		–277		–12·02		+16		+871		– 0·55		King	475

* Cell counts on this case unreliable, as all samples showed auto-agglutination.
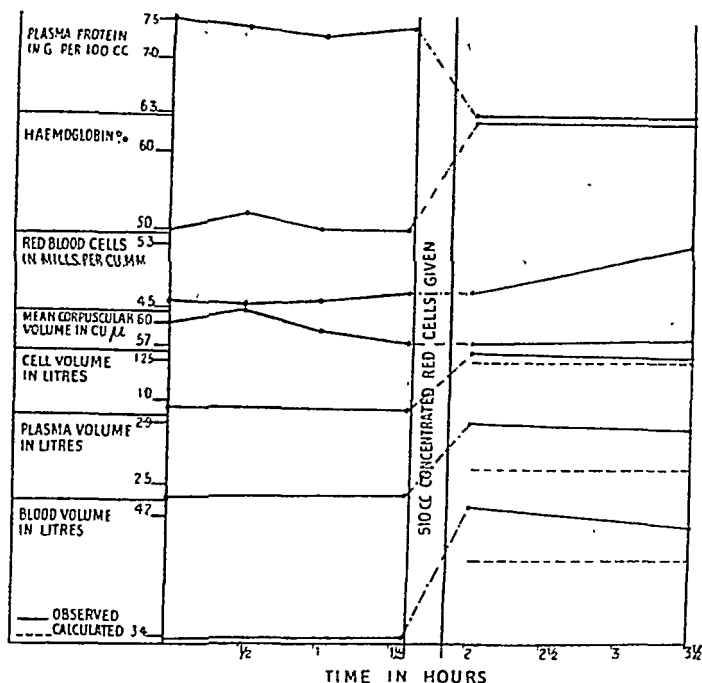
FIG. 3.—Changes in serum protein, hæmoglobin, red cells, mean corpuscular volume, cell volume, plasma volume, and blood volume in a patient with hypochromic anæmia after receiving 510 c.c. concentrated red cells. Hæmodilution occurred.
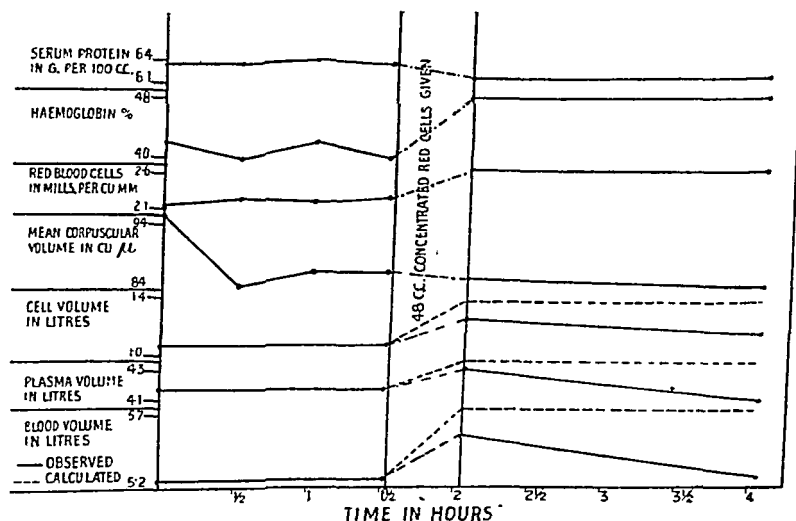
FIG. 4.—Changes in serum protein, hæmoglobin, red cells, mean corpuscular volume, plasma volume, and blood volume in a patient with ? hæmolytic anæmia after receiving 480 c.c. concentrated red cells. Hæmoconcentration occurred.

were added. Such loss of fluid and shrinkage of the cells will reduce the value of the packed red cells, hence the total cell volume is less than calculated.

DISCUSSION.

It is not claimed that the methods employed are of sufficient accuracy to permit any weight to be attached to small differences observed following a particular experimental procedure. The consistency of mean corpuscular hæmoglobin before and after bleeding suggests, however, that the results obtained can be accepted as approximating to correct values. It is easier to interpret the results of removing a constant amount of blood from normal individuals than it is to interpret the findings following transfusion of stored red cells into a small mixed group of anæmias. In the latter neither the character of the transfused fluid nor the character of the blood in the recipient was constant. These observations, however, demonstrate that considerable and variable changes in plasma volume may occur and must of necessity affect the hæmoglobin level following either blood loss or administration. It is therefore essential to appreciate that it is unwise to assess the significance of changes in hæmoglobin level unless plasma volume is also measured. There is some rough agreement between figures expressing alteration in plasma volume, hæmoglobin, red-cell count, and mean corpuscular volume in the majority of cases, though it breaks down in one or two instances. For instance, the plasma volume in Case 1 after bleeding shows an overdilution of 612 c.c., but a smaller observed fall in red-cell count than Case 3 which dilutes less. This discrepancy might be accounted for by postulating the liberation of a small store of red cells to meet overdilution, but there is no satisfactory evidence that such stores exist in man [Brown *et al.*, 1942].

In spite of such a discrepancy these figures clearly indicate, however, that in certain individuals following the administration or loss of blood under standard conditions fluid may pass into the circulation from the tissues or *vice versa*. The rate at which the fluid shift occurs and its time relationship to the blood loss or administration is also variable. Some of the fluid passing into the circulation remains in the plasma, while some is clearly absorbed by the red cells since hæmodilution may be associated with an increase in mean corpuscular volume and in total cell volume. The present observations offer no explanation of why dilution occurs in some individuals and not in others. There was no obvious difference in age or mode of life between the subjects who diluted markedly after bleeding and those who showed little or no dilution. Case 3 (Table III), who diluted well, had a severe vasovagal attack directly after bleeding, while Case 2, who diluted to the same degree, did not. The nature of the fluid passing into the circulation from the tissues is also not clear.

The fact that in the majority of cases there is a maintained increase in mean corpuscular volume suggests that the entering fluid may be of lower osmotic pressure than the plasma. This is supported by the fact that definite lowering of serum protein was obvious in three instances, and that every change in serum protein noted, even though in some instances small, was a decrease. In no instance was there an increase in serum proteins. The possible occurrence of such rapid changes in plasma volume clearly makes it difficult to assess the significance of hæmoglobin estimations following either blood loss or blood gain unless serial estimations are made over a relatively long period. In Case 8 (Table III), for instance, 540 c.c. of blood were removed, leaving the hæmoglobin and red-cell count unchanged.

Conclusions.

1. In assessing the significance of changes in hæmoglobin, total red cells, mean corpuscular volume, and serum protein in any individual. the importance of possible changes in plasma volume must be considered. A fall in hæmoglobin or red cells may be due to dilution as well as to actual blood loss. Blood loss may occur without affecting the hæmoglobin level.

2. In estimating the amount of fluid that has passed from the tissues into the circulation, the amount absorbed by the red cells is significant and must be taken into account.

3. There is considerable variation in the response of different individuals to the removal of 540 c.c. of blood or to the administration of 450–520 c.c. of concentrated red cells.

We are grateful to Dr. C. M. Scott, Imperial Chemical Industries. for a supply of Evans blue dye.

Summary.

Observations on the changes in red cells, hæmoglobin, mean corpuscular volume, serum protein, and plasma volume in a two-hour period following the removal of 540 c.c. of blood were made on 8 normal subjects and following the transfusion of concentrated red cells on 8 anæmic subjects. Following the removal of blood in one instance the plasma volume was that calculated, in others dilution or over-dilution were noted. Dilution following blood loss was associated with an increase in mean corpuscular volume, suggesting that some of the fluid passing in from the tissues was absorbed by the red cells. No increase in serum protein was noted. In some cases there was a significant fall. In assessing the significance of changes in hæmoglobin, total red-cell count, mean corpuscular volume, and serum protein in any individual, the possibility of changes in plasma volume must

therefore be considered. A fall in hæmoglobin or red cells may be due to dilution as well as to actual blood loss. Blood loss may occur without affecting the hæmoglobin level. In estimating the amount of fluid that has passed from the tissues into the circulation, the amount absorbed by the red cells is significant and must be taken into account. There is clearly considerable variation in the response of different individuals to the removal of 540 c.c. of blood or to the administration of 400–500 c.c. of concentrated red cells.

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The fact that in the majority of cases there is a maintained increase in mean corpuscular volume suggests that the entering fluid may be of lower osmotic pressure than the plasma. This is supported by the fact that definite lowering of serum protein was obvious in three instances, and that every change in serum protein noted, even though in some instances small, was a decrease. In no instance was there an increase in serum proteins. The possible occurrence of such rapid changes in plasma volume clearly makes it difficult to assess the significance of hæmoglobin estimations following either blood loss or blood gain unless serial estimations are made over a relatively long period. In Case 8 (Table III), for instance, 540 c.c. of blood were removed, leaving the hæmoglobin and red-cell count unchanged.

Conclusions.

1. In assessing the significance of changes in hæmoglobin, total red cells, mean corpuscular volume, and serum protein in any individual, the importance of possible changes in plasma volume must be considered. A fall in hæmoglobin or red cells may be due to dilution as well as to actual blood loss. Blood loss may occur without affecting the hæmoglobin level.

2. In estimating the amount of fluid that has passed from the tissues into the circulation, the amount absorbed by the red cells is significant and must be taken into account.

3. There is considerable variation in the response of different individuals to the removal of 540 c.c. of blood or to the administration of 450–520 c.c. of concentrated red cells.

Summary.

Observations on the changes in red cells, hæmoglobin, mean corpuscular volume, serum protein, and plasma volume in a two-hour period following the removal of 540 c.c. of blood were made on 8 normal subjects and following the transfusion of concentrated red cells on 8 anæmic subjects. Following the removal of blood in one instance the plasma volume was that calculated, in others dilution or over-dilution were noted. Dilution following blood loss was associated with an increase in mean corpuscular volume, suggesting that some of the fluid passing in from the tissues was absorbed by the red cells. No increase in serum protein was noted. In some cases there was a significant fall. In assessing the significance of changes in hæmoglobin, total red-cell count, mean corpuscular volume, and serum protein in any individual, the possibility of changes in plasma volume must

therefore be considered. A fall in hæmoglobin or red cells may be due to dilution as well as to actual blood loss. Blood loss may occur without affecting the hæmoglobin level. In estimating the amount of fluid that has passed from the tissues into the circulation, the amount absorbed by the red cells is significant and must be taken into account. There is clearly considerable variation in the response of different individuals to the removal of 540 c.c. of blood or to the administration of 400–500 c.c. of concentrated red cells.

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METABOLISM OF LIVER SLICES AFTER BURNING.[1] By E. J. CLARK and R. J. ROSSITER. From the Department of Biochemistry, Oxford.

THERE are two main views as to the cause of the systemic effects of serious burns. The first is that of a specific burn toxin, favoured, for example, by Bardeen [1898], Pfeiffer [1905], Vogt [1912], and Vaccarezza [1922], and, more recently, by Robertson and Boyd [1923] and Wilson *et al.* [1937]; the other is that the ill effects are secondary to the loss of fluid and protein from the blood into the burned tissues [Underhill, 1927; Underhill *et al.*, 1930; Blalock, 1931; and Harkins, 1934, 1935. See also Rossiter, 1943, for a review]. Since the tissue slice technique has, in the past, been useful for investigating the metabolic effects of certain known toxins [Cross and Holmes, 1937; Dawson and Holmes, 1939; and Holmes, 1939, for summary], it seemed of interest to apply this method to the problem of burn toxæmia, with the double purpose of seeing if any metabolic process were acutely affected and of comparing such changes as might occur with previously established effects of undoubted toxæmia on metabolism.

The liver was chosen for these experiments because of the many reports of impaired liver function after burns [Boyce and McFetridge, 1938 *b*; Wolff *et al.*, 1940; Thompson and Wilkinson, 1940; and Abbott and Holden, 1942], and also of histological liver damage [Bardeen, 1897; Pfeiffer, 1905; Olbrycht, 1924; and more recently Wilson *et al.*, 1938; McClure, 1939; Belt, 1939; Brins and Hartmann, 1941; and Wells *et al.*, 1942]. It was thought that changes in liver metabolism might prove a more sensitive test for liver damage than the microscopic appearance. But it must be remembered that the histological damage and impaired liver function may be produced by tannic acid used in treatment [Wells *et al.*, 1942; Cameron *et al.*, 1943; Barnes and Rossiter, 1943; Clark and Rossiter, 1943; Erb *et al.*, 1943; Robinson and Graessle, 1943; and Forbes and Evans, 1943]. Anæsthetics, too, may affect liver function tests [Boyce and McFetridge, 1938 *a*, and Thompson and Wilkinson, 1940]; in the present work, complication by anæsthesia was

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[1] Experiments performed under the direction of Professor R. A. Peters, F.R.S., on behalf of the Burns Sub-committee of the War Wounds Committee, Medical Research Council. Report submitted September 1943.

avoided, as far as possible, by anæsthetising all controls for the same period as burned animals.

Measurements were made of the Q_{O_2}, R.Q. and the increase in O_2 uptake in the presence of either alanine or sodium butyrate in phosphate buffer, and of the Q_{O_2}, $Q_G^{O_2}$, $Q_G^{N_2}$ and aerobic synthesis of glycogen from glucose in bicarbonate buffer. Thus, in addition to the usual respiratory metabolism, stages in the metabolism of protein, fat, and carbohydrate were investigated The animals were killed 4 hours (at the height of the hæmoconcentration) and 24 hours after burning. This should cover for the rabbit, the equivalent period of the acute toxæmic phase seen in man.

METHODS.

Manometric measurements were made with Warburg constant volume manometers. The O_2 uptake and R.Q. experiments were performed in phosphate buffer pH 7·3. CO_2 production was determined by difference, bound CO_2 being liberated both before and after the experimental period. Glycolysis was measured manometrically. $Q_G^{O_2}$ by the Warburg 2-bottle method. The following physiological salt mixture was used:—

NaCl .	.	.	0·8 per cent.	96 ml.
KCl .	.	.	1·0 per cent.	3 ml.
CaCl₂	.	.	1·12 per cent.	2 ml.

For experiments in phosphate buffer, 20 ml. buffer (Na_2HPO_4 8·85 g. in 250 ml. plus 12·5 ml. N = 1 HCl) was added to 100 ml. medium immediately before use. For glycolysis, 20 ml. 1·26 per cent. $NaHCO_3$ was added to the mixture of sodium and potassium chlorides. This was equilibrated with a gas mixture containing 5 per cent. CO_2 before the further addition of the calcium chloride. Glucose was added to make the final concentration 0·5 per cent. In the glycogen synthesis experiments, the medium was the same as for glycolysis; 1 ml. 60 per cent. KOH was added to the bottles, either immediately before or immediately after incubation. The difference in glycogen content was reckoned as glycogen synthesised. Glycogen was determined by a modification of the method of Good et al. [1933], the sugar formed being estimated by the ferricyanide method. All experiments were done in an atmosphere of O_2, except glycolysis, where the appropriate gas mixture was used. Slices were cut with a moist safety-razor blade and floated off in buffered salt solution. After the experiment they were dried to constant weight. The slices for glycogen estimations were cut with a dry blade and rapidly weighed on a torsion balance. Determinations were done either in duplicate or triplicate.

EXPERIMENTAL.

Young rabbits (500–1000 g.) were clipped closely with scissors, anæsthetised with ether and burned by dipping the back (approximately 1/3 body surface) into water at 70° C. for $\frac{1}{2}$ minute. Previous experiments with guinea-pigs indicated that this method of burning produced damage equivalent to a 1-minute application at 60–65° C. of the burning iron previously described [Leach, Peters, and Rossiter, 1943]. There was marked œdema and increase in red blood cell count within 2–4 hours (Table I). This increase had disappeared by the end of 24 hours and the œdema was much less. After either 4 or 24 hours the animal was stunned by a sharp blow on the head, decapitated, bled, and the liver rapidly dissected out for cutting the slices. The animals usually survived this method of burning, although an occasional one died before observations could be made. With more severe burns (higher temperature or greater body surface), the animal usually died within 4 hours. The burn was in fact the most severe non-fatal burn of this type that could be consistently produced in the animals used. The animals were all starved for a period of 24 hours immediately before killing, regardless of the time of burning.

TABLE I.—MEAN RED CELL COUNT (10^6 CELLS/MM.^{3}) IN RABBITS.

Treatment.	No. animals.	R.B.C. before.	R.B.C. after.	Mean change R.B.C. ($\pm$ S.E. mean).
Anæsthetised only. Sample taken 2–4 hours later.	11	5·3	5·4	+0·1 ($\pm$ 0·1)
Burnt. Sample taken 2–4 hours later.	16	5·7	7·5	+1·8 ($\pm$ 0·2)
Burnt. Sample taken 24–26 hours later.	4	5·8	5·8	0·0 ($\pm$ 0·4)

RESULTS.

The results are summarised in fig. 1. In phosphate buffer (Table II) there was no significant change in the oxidation of alanine or sodium butyrate either 4 or 24 hours after burning. There was no change in Q_{O_2} after 4 hours, but after 24 hours a slight, but nevertheless significant ($t = 3·42$; for $P = 0·01$, $t = 3·36$), rise was observed. The R.Q. was significantly ($t = 2·22$; for $P = 0·05$, $t = 2·20$) lower 4 hours after burning, but had returned to normal within 24 hours.

In bicarbonate buffer (Table III) there was no change in Q_{O_2} or $Q_G^{O_2}$ either 4 or 24 hours after burning, but the $Q_G^{N_2}$ rose slightly ($P = 0.085$) within 4 hours and returned to normal again by 24 hours. Liver tissue from animals that had merely been anæsthetised formed
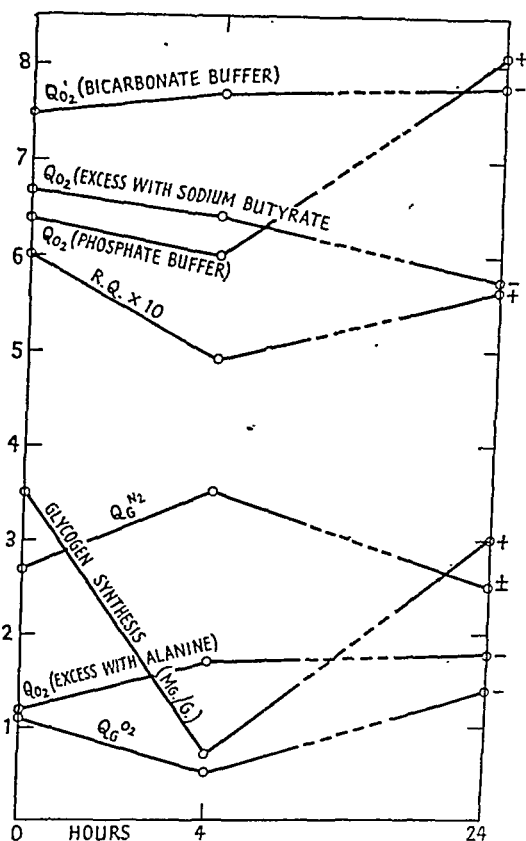
FIG. 1.—Metabolism of rabbit liver slices. Abcissa, time after burning. Animals at time 0 were anæsthetised only.   +, differences observed were significant (P < 0.05).   ±, differences were of doubtful significance (P = 0.1–0.05).   −, differences were not significant (P > 0.1).

glycogen readily from glucose (Table IV), but 4 hours after burning this ability had greatly diminished, to return again within 24 hours. This greater synthesis with the control animals cannot be accounted for by the greater initial liver glycogen because, in general, the greater the initial glycogen value, the poorer was the synthesis; also, the livers of the animals killed 24 hours after burning with a glycogen content not significantly different from those killed after 4 hours, formed glycogen quite normally.

TABLE II.—EXPERIMENTS IN PHOSPHATE BUFFER. RABBIT LIVER SLICES. RABBIT STARVED 24 HOURS BEFORE BEING KILLED.

Q_{O_2}.	Q_{O_2} in presence of alanine. (0·4 per cent.).	Excess due to alanine.	Q_{O_2} in presence of sodium butyrate (0·4 per cent.).	Excess due to sodium sodium butyrate.	R.Q.
		A. Anæsthetised only.			
6·1	8·4	2·3	15·1	9·0	0·67
5·5	6·0	0·5	10·7	5·2	0·67
6·3	6·9	0·6	13·5	7·2	0·48
6·7	8·4	1·7	13·5	6·8	0·49
5·9	6·4	0·5	12·1	6·2	0·71
7·7	9·6	1·9	13·7	6·0	0·56
Mean 6·4	7·6	1·2	13·1	6·7	0·60
		B. Burnt. Killed 4 Hours later.			
5·9	6·1	0·2	10·2	4·3	0·55
6·9	7·9	1·0	13·5	6·6	0·56
5·1	7·5	2·4	12·3	7·2	0·48
5·4	7·4	2·0	12·6	7·2	0·43
4·6	8·2	3·6	13·1	8·5	0·57
7·7	9·3	1·6	14·4	6·7	0·47
6·3	7·7	1·4	10·6	4·3	0·38
Mean 6·0	7·7	1·7	12·4	6·4	0·49
		C. Burnt. Killed 24 Hours later.			
8·1	8·5	0·4	13·2	5·1	0·57
7·0	9·4	2·4	12·8	5·8	0·58
8·6	10·8	2·2	15·2	6·6	0·52
8·7	10·7	2·0	13·9	5·2	0·61
Mean 8·1	9·9	1·8	13·8	5·7	0·57

DISCUSSION.

There is evidently a general metabolic disturbance 4 hours after burning, as is shown by the lowered R.Q., increased anaerobic glycolysis and impaired ability to form glycogen from glucose, which returns to normal after 24 hours. These changes coincide with the hæmoconcentration, so they may possibly be part of the shock reaction. The main points about the changes are that they are not of very striking extent, apart from the failure of glycogen synthesis, and that they are certainly reversible; there was no evidence of persisting liver damage, the only abnormality found after 24 hours being a rise in oxygen uptake

in phosphate buffer (though not in bicarbonate). As the most severe burn compatible with survival was used, this indicates that, in the rabbit, recovery from such a burn is not associated with persistent metabolic disturbances. Comparison of the present results with those

TABLE III.—EXPERIMENTS IN BICARBONATE BUFFER. RABBIT LIVER SLICES. RABBIT STARVED 24 HOURS BEFORE BEING KILLED.

	Q_{O_2}.	$Q_G^{O_2}$.	$Q_G^{N_2}$.
A. Anæsthetised only.			
	11·0	2·9	3·0
	11·1	1·8	3·1
	6·0	0·1	2·9
	6·8	0·0	··
	5·3	0·0	2·0
	7·2	2·8	3·6
	5·3	0·0	1·7
Mean	7·5	1·1	2·7
B. Burnt. Killed 4 Hours later.			
	8·3	0·4	3·4
	7·5	0·0	3·7
	6·7	0·2	4·3
	8·2	1·3	2·6
	10·3	3·9	3·6
Mean	8·2	1·2	3·5
C. Burnt. Killed 24 Hours later.			
	6·4	0·0	2·5
	6·4	1·7	2·8
	8·3	1·4	2·3
	10·2	2·5	2·2
Mean	7·8	1·4	2·5

of Cross and Holmes [1937] on diphtheritic toxæmia in rabbits is, consequently, not of great significance, since the latter authors used lethal doses of toxin. It is noteworthy, however, that the failure of glycogen synthesis after burning occurred under the conditions in which Cross and Holmes found no failure of synthesis, viz. in the presence of excess glucose. The reduction of oxygen uptake in the absence of substrate, after 24 hours diphtheritic toxæmia, coincides in time with the rise of oxygen uptake and return of other metabolic processes to normal after burning; it may be observed, however, that some liver poisons, *e.g.* phosphorus, carbon tetrachloride, and chloroform, raise the Q_{O_2} of liver slices [Meier and Thoenes, 1933; Ennor, 1942]. The reduction of

R.Q. without substrate after burning is in the same direction as that found by Cross and Holmes in toxæmia, but again the time relations differ; in the present observations, too, the variability of individual R.Q.'s makes the changes observed in the means barely significant.

TABLE IV.—SYNTHESIS OF GLYCOGEN FROM GLUCOSE BY RABBIT LIVER SLICES. RABBIT STARVED 24 HOURS BEFORE BEING KILLED. GLUCOSE CONCENTRATION 0·5 PER CENT. BICARBONATE BUFFER. ATMOSPHERE, 95 PER CENT. O₂, 5 PER CENT. CO₂. INCUBATION TIME, 1 HOUR.

Glycogen before incubation (mg./g.).	Glycogen after incubation (mg./g.).	Glycogen synthesised (mg./g./hr.).
A. Anæsthetised only.		
1·2	5·4	4·2
6·1	9·1	3·0
7·8	11·3	3·5
4·8	7·8	3·0
3·8	7·6	3·8
Mean 4·7	8·2	3·5
B. Burnt. Killed 4 Hours later.		
0·4	1·5	1·1
0·6	1·2	0·6
0·2	0·4	0·2
2·7	3·2	0·5
2·9	4·3	1·4
0·9	2·1	1·2
0·8	0·9	0·1
Mean 1·2	1·9	0·7
C. Burnt. Killed 24 Hours later.		
1·5	4·5	3·0
3·6	6·9	3·3
0·8	3·6	2·8
0·9	3·7	2·8
Mean 1·7	4·7	3·0

The metabolic changes in the liver after non-fatal burns are distinct, both quantitatively and in time relations, from the changes found in diphtheritic toxæmia, but do not exclude the possibility of a different toxic agent; equally, however, they may be secondary to the changes accompanying the diminished blood volume associated with the period of shock, *e.g.* lowered oxygen partial pressure, acidosis and changes in the fluid and electrolyte balance. In this connection, it is of interest

to point out that decreased oxygen partial pressure [Ostern *et al.*, 1939; Lee and Richter, 1940], acidosis [Fantl *et al.*, 1942], and increased chloride concentration [Eadie, 1927, and Davenport, 1926]—all conditions which occur following burns—have been reported either to increase glycogen breakdown or to decrease glycogen synthesis by liver enxyme systems "*in vitro.*"   It must be stressed, however, that in these experiments changes were made in the medium in which the activity of the enzyme systems were measured.   In the experiments reported in this paper, the observations were made on liver slices suspended in a standard medium.   The difference in the metabolism observed are, therefore, effects persisting "*in vitro,*" of conditions which, before death, prevailed "*in vivo.*"   In general, it is felt by the writers that these changes are in fact secondary to the reduced blood volume;   indirect evidence bearing on this is the coincidence in time of the metabolic changes and the hæmoconcentration.

SUMMARY.

1. Anæsthetised rabbits burnt at 70° C. for $\frac{1}{2}$ minute (area of burn 1/3 body surface) show a rise in red blood cell count within 2–4 hours, which has fallen to normal by 24 hours.

2. There is no change in the ability of the liver to oxidise alanine or sodium butyrate either 4 or 24 hours after burning;   there is also no change in the $Q_G^{O_2}$ and Q_{O_2} measured in bicarbonate buffer.

3. After 4 hours there is a fall in the R.Q. (measured in phosphate buffer), rise in the $Q_G^{N_2}$, and fall in the ability of the liver to form glycogen from glucose;   these have all returned to normal within 24 hours.

4. After 24 hours there is a slight rise in the Q_{O_2} measured in phosphate buffer, which is not observed after 4 hours.

5. Implications of these results are discussed and it is concluded that they are in all probability secondary to the circulatory changes following the burn.

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CARBOHYDRATE METABOLISM AFTER BURNING.[1] By E. J. CLARK and R. J. ROSSITER. From the Department of Biochemistry, Oxford.

AFTER burning there is a rise in blood sugar, both in man [Underhill *et al.*, 1923; Davidson, 1925; McIver, 1933; and Lambret *et al.*, 1936], and in experimental animals [Schreiner, 1926; Greenwald and Eliasberg, 1926; Lundberg, 1929; Beard and Blalock, 1931; Slocum and Lightbody, 1931; and Stolfi, 1936]. The reports of this hyperglycæmia are scattered in the literature and, as a result, they do not seem to be generally known to those responsible for the treatment of burn casualties. In the present study we have established that there is a rise in blood-sugar after burning in both the rabbit and the rat, and an attempt has been made to investigate the mechanism whereby this hyperglycæmia is produced.

In rabbits, the rise in blood sugar after burning is greater in well-fed than in starved animals, and in animals deprived of food for 24 hours there is a fall in liver glycogen after burning. In the rat, there is, in addition, a transient rise in blood lactic acid and a fall in muscle glycogen. These changes cannot be produced in an adrenalectomised animal, which suggests that they may be part of an adrenaline response, especially since they can all be imitated by adrenaline injections. Additional evidence in favour of the view is the fact that burning reduces the vitamin C content of the adrenal gland in rabbits, presumably as a result of some abnormal adrenal activity. There is no change in the vitamin C content of the liver.

On the other hand, Clark and Rossiter [1943] have reported that, in rabbits after burning, there is a transitory deficiency (roughly corresponding in time with the hyperglycæmia) in the ability of liver slices to form glycogen from glucose; this ability has now been shown to be normal after an adrenaline injection which can produce a similar rise in blood sugar. Also, after adrenaline injections there is a rise in liver glycogen in both the rat and the rabbit [see Cori, 1931, for references], while in the present experiments, although adrenaline injections into rats

produce a rise in liver glycogen, burning does not cause this rise and, if anything, causes a fall; with rabbits, this fall is very striking. There must be, therefore, some other factor which prevents the normal deposition of liver glycogen, even in the presence of a high blood lactic acid. There is thus the possibility of there being two mechanisms contributing to the burn hyperglycæmia: an adrenaline response (mechanism *a*) and an additional mechanism, which either stimulates liver glycogenolysis or depresses glycogenesis (mechanism *b*). Evidence for each of these is set out later, in the discussion. Attempts to demonstrate an increased "*in vitro*" glycogenolysis, either by phosphorylase or amylase activity, in liver brei have been unsuccessful; nor is there any change in blood amylase, which some workers suggest [Cori and Cori, 1938, and Lee and Richter, 1940] is identical with liver amylase.

The observations of Guest and Rawson [1941] that, for the rat, acidosis produced by ammonium chloride injection gives a rise in blood sugar of the same order as had been observed after burning, suggested the possibility that burn hyperglycæmia might be secondary to a diminished alkali reserve. It has been found, however, that the amount of ammonium chloride necessary to produce changes in blood sugar equivalent to those observed after burning causes a marked decrease in the plasma alkali reserve, whereas the decrease observed after burning is, by comparison, trifling. Also sodium bicarbonate injections fail to influence significantly the blood-sugar rise which follows burning. These experiments have, therefore, led to the conclusion that the changes in carbohydrate metabolism, which follow burning, are not secondary to changes in the plasma acid—base balance.

A practical point is whether the hyperglycæmia in any way helps the animal to withstand the ill-effects of burning. It has been shown that well-fed animals resist burning no more successfully than similar animals starved for 24 hours. It must be remembered, however, that after burning the carbohydrate stores are depleted, and that an adequate diet, rich in carbohydrate, will help to replenish the stores and also, perhaps, spare protein [Clark, Peters, and Rossiter, 1943].

METHODS.

Animals.—For experiments of Section A rabbits (500–1000 g.) and of Section B rats (100–150 g.) were used. The rats used for the glycogen experiments were all of the same strain.

Blood Sugar.—By method of Hagedorn and Jensen. [1923]

Lactic Acid.—By method of Friedemann *et al.* [1927].

Glycogen.—By method of Good *et al.* [1933].

Phosphate.—By method of Fiske and Subbarow [1925].

Vitamin C.—Determined in a metaphosphoric acid extract by the method of Harris and Olliver [1942].

Glycogen Synthesis.—This was measured as described previously [Clark and Rossiter, 1943].

Serum Amylase.—By method of Lagerlöf [1942].

Alkali Reserve.—This was measured by displacement of CO_2 from plasma equilibrated with 5 per cent. CO_2 in a Warburg manometer.

Liver Phosphorolysis and Amylolysis.—This was determined by the method of Lee and Richter [1940].

The adrenaline preparation was that supplied by Messrs. Park Davis Ltd.

Blood Samples were taken from a marginal ear vein, in the case of rabbits, and after decapitation in the case of rats, except for the alkali reserve experiments, when blood samples were taken by heart puncture during ether anæsthesia, and centrifuged under paraffin as described by Van Slyke and Cullen [1917]. Oxalate was used as an anti-coagulant.

Glycogen Samples.—For glycogen estimations in rabbit liver, samples were taken immediately after death and placed in potash. With the rat, the whole liver or whole carcass was immediately placed in potash. No further precautions were taken to retard glycolysis.

Method of Burning.—The rabbits were clipped closely with scissors, anæsthetised with ether and burned by dipping the back (approximately 1/3rd of the body surface) into water at 70° C. for 30 seconds. The rats were anæsthetised with ether, and the back (approximately 1/3rd of the body surface) dipped into water at 80° C. for 30 seconds. This method of burning produced damage equivalent to a 1-minute application at 60–65° C. of the burning iron previously described [Leach, Peters, and Rossiter, 1943]. In both cases control animals were similarly anæsthetised but not burned.

Adrenalectomy was performed by the technique of Firor and Grollman [1933].

Results.

A. Experiments with Rabbits.

1. *Blood Sugar.*—The rise in blood sugar following burning, in rabbits, is shown in fig. 1. The rise was greater with animals that had been well fed than with those deprived of food for 24 hours, and was similar in duration to that observed after adrenaline (0·3 mg. kg.) injections. By the twenty-fourth hour after burning, the blood sugar had returned to normal and, in control animals that had been anæsthetised only, there was but a small rise. The values of P obtained by Fisher's "*t*" test, show that the rise observed in animals 4 hours after burning (in italics), is significantly different from that in the control group, in both well-fed and 24 hour-starved animals. Details of these experiments will be found in the Appendix, Table I.

2. *Formation of Glycogen from Glucose.*—It has previously been shown that liver slices from rabbits, killed 4 hours after burning, have

an impaired ability to form glycogen from glucose [Clark and Rossiter, 1943].   Since the rise in blood sugar following burns was similar to that observed after a suitable injection of adrenaline (0·3 mg./kg.), it seemed of interest to test the glycogen-forming power of liver slices from animals killed 4 hours after the injection of such an hyperglycæmic dose of adrenaline.   Table II of the Appendix shows that, whereas liver slices from animals burned 4 hours previously had a significantly deficient
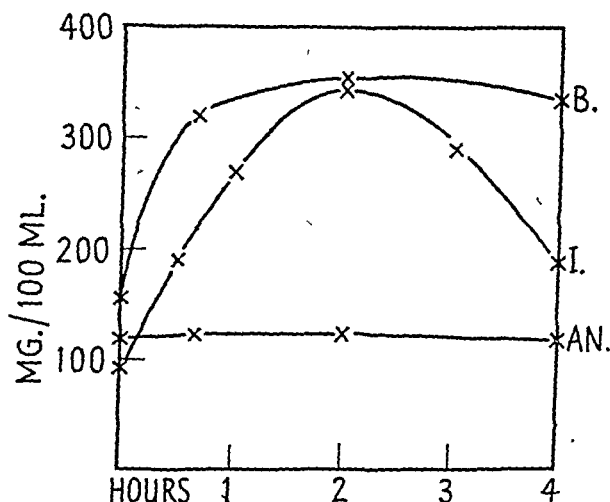

FIG. 1.—Blood sugar of well-fed rabbits.

AN, anæsthetised only.   B, burned for 30 seconds at 70° C.   I, injected with 0·3 mg./kg. adrenaline subcutaneously.

glycogen synthesis, slices from adrenaline-injected animals formed glycogen perfectly normally.

   3. *Liver Glycogen after Burning.*—In animals that had been fasted for 24 hours, the glycogen of the liver, both 4 and 24 hours after burning, was consistently lower than that of control animals that had been anæsthetised only (Appendix, Table III).

   4. *Glycogenolysis "in vitro."*—It is now known that there are two mechanisms whereby liver brei destroys glycogen—a phosphorylating and an amylolytic mechanism [Cori and Cori, 1938, and Ostern *et al.*, 1939].   These processes were separated by the method described by Lee and Richter [1940].   The system used was as follows: 4 ml. brei (equal volumes cooled liver and ice-cold water), 0·2 ml. 0·1 per cent. adenylic acid, 0·2 ml. magnesium chloride (1 mg. magnesium/ml.), 1·0 ml. M/2 sodium fluoride, 1·0 ml. M/9 phosphate buffer pH 7, 6·0 ml. water (containing 100 mg. glycogen).

   Table IV of the Appendix shows that, whereas the glycogen breakdown by amylase and phosphorylase activity is on the average slightly

greater with the brei from the burned animals, the scatter was so great that the differences observed were not statistically significant. Application of the "t" test to the difference between the means of the control and experimental groups in the phosphorolysis experiments after 30 minutes gives $t = 0.727$, corresponding to $P = 0.49$; similarly for amylolysis after 60 minutes, $t = 0.57$, corresponding to $P = 0.59$.

5. *Serum Amylase.*—It was possible that the increased glycogenolysis following burning might be due to an increase in the quantity of amylase in the serum. A fall in blood volume is known rapidly to mobilise protein reserves, and it is possible that such a mobilisation from the pancreas would result in an increase in serum amylase. As has been pointed out already, suggestions have been made that serum amylase and liver amylase are identical.

The amylase activity was determined by the method of Lagerlöf [1942] and has been expressed as the number of mg. maltose formed from the breakdown of 1 ml. of a 1 per cent. starch solution (containing 10 mg. starch) by 1 ml. of a 1 : 4 dilution of serum after incubation at 38° C. for one hour. Table V of the Appendix shows that, far from there being an increase in the serum amylase, there was, if anything, a slight decrease. This may possibly be associated with loss of protein to the burned area.

6. *Vitamin C in Adrenal Gland and Liver.*—There was a significant fall in the ascorbic acid content of the adrenal gland, both 4 and 24 hours after burning, but there was no change in the ascorbic acid content of the liver (Appendix, Table VI).

B. *Experiments with Rats.*

1. *Blood Sugar.*—With rats also, there was a rise in blood sugar one hour after burning which, though significantly reduced, had not returned to normal within three hours (fig. 2, *a*). With control animals, anæsthetised only, there was no rise. A similar rise in blood sugar was observed after injections of adrenaline (0·05–0·1 mg./kg.), while a larger dose (0·2–0·5 mg./kg.) caused a greater response. Immediately after adrenalectomy there was little change in the blood sugar, and when such an adrenalectomised animal was burned there was still only a very small rise. Thus in the rat the adrenal glands are necessary for the hyperglycæmia which occurs after burning. For details of these experiments see Appendix, Table VII. Table VIII of the Appendix shows that, unlike the rabbit, the well-fed rat (starved for 24 hours and then fed 600 mg. glucose/100 g. body-weight by stomach tube 3 hours before burning) did not give a greatly increased hyperglycæmic response after burning.

2. *Lactic Acid.*—After burning, there was also a significant rise in blood lactic acid, which had fallen to normal levels by the end of 3 hours
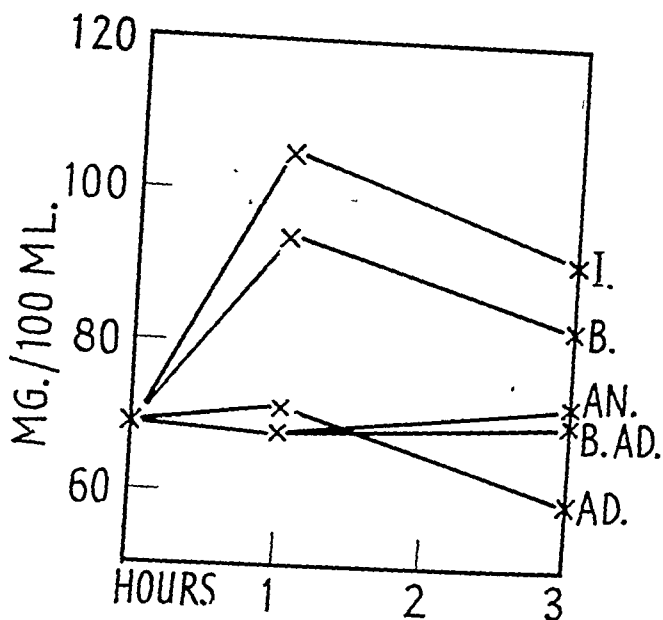
Clark and Rossiter

FIG. 2, *a*, *b*, and *c*.—*a*, blood sugar. *b*, blood lactic acid. *c*, carcass glycogen of rats starved for 24 hours.

AN, anæsthetised only. B, burned for 30 seconds at 80 C. I, injected with 0·05–0·1 mg. kg. adrenaline subcutaneously. Adr, adrenalectomised only. B.Adr, adrenalecto-mised and burned as above.
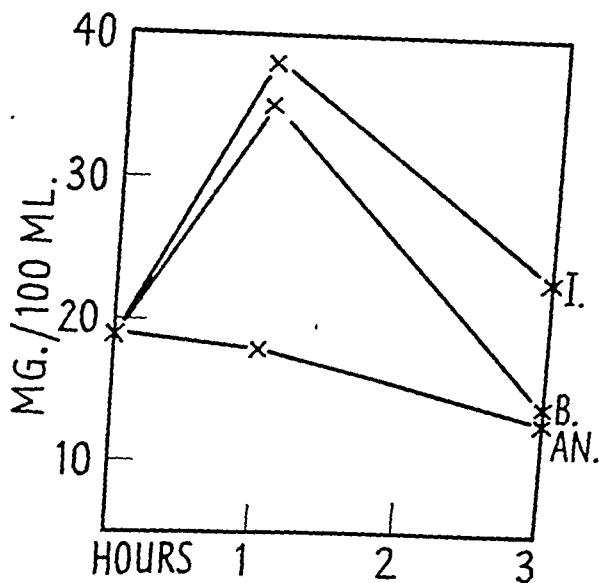

FIG. 2, *b*.

(fig. 2, b). This rise was similar, both in magnitude and duration, to the well-known increase in blood lactic acid which follows adrenaline injections (0·05–0·1 mg./kg.). For larger doses of adrenaline (0·2–0·5 mg./kg.), the rise in lactic acid was greater. Again, control animals, anæsthetised only, showed no significant change (for details see Appendix, Table IX).

3. *Carcass Glycogen*.—The glycogen content of the carcass (chiefly muscle glycogen) had fallen significantly one hour after burning, while
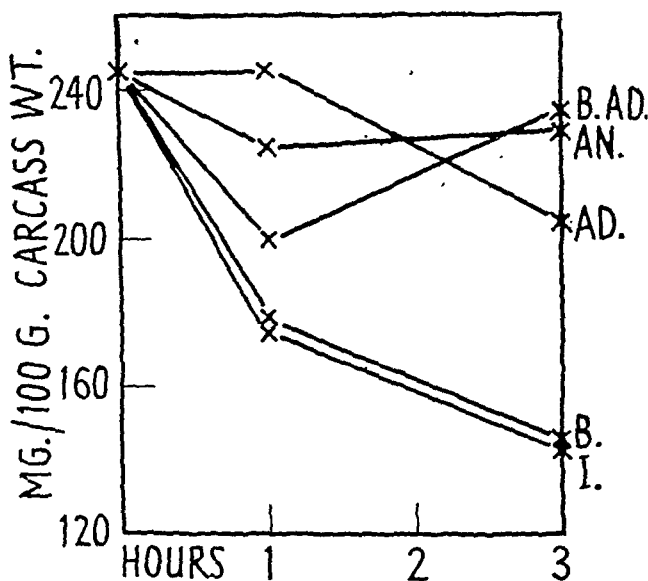
FIG. 2, c.

3 hours later it had fallen still further (fig. 2, c). A very similar fall was observed with animals injected with 0·05–0·1 mg./kg. adrenaline, a dose which gave a rise in blood sugar and a rise in blood lactic acid very similar to that seen after burning. A larger injection of adrenaline (0·2–0·5 mg./kg.) gave a greater fall in carcass glycogen. Merely anæsthetising the animals, or removing the adrenal glands, gave no such changes in the carcass glycogen stores. When adrenalectomised animals were burned, there was a slight fall in carcass glycogen one hour later. This fall was, however, much smaller than that found after burning, or after adrenaline injections, and was not observed in adrenalectomised animals 3 hours after burning (Appendix, Table X).

4. *Liver Glycogen*.—Under the conditions of these experiments (starved for 24 hours), all the animals showed a very constant liver glycogen of about 5–7 mg./100 g. carcass weight (Appendix, Table XI). This was slightly increased ($P = 0.07$) by an adrenaline injection of

0·05–0·1 mg./kg. and definitely increased (P = 0·05) by the larger dose (0·2–0·5 mg./kg.). It is noteworthy that, although adrenaline produced this increase, there was no change after burning.

5. *Is Burn Hyperglycæmia Produced by Acidosis?*—Injection of 5 ml. isotonic ammonium chloride intraperitoneally caused a rise in blood sugar very similar to that observed after burning (Appendix, Table XII). This ammonium chloride injection also caused a marked fall in the plasma alkali reserve, but burning, which produced a similar rise in blood sugar, only resulted in a very slight change in the alkali reserve. In addition, animals were injected either with 5 ml. isotonic sodium bicarbonate or 5 ml. isotonic sodium chloride, burned immediately, and the blood sugar determined one hour later. Table XIII of the Appendix shows that there was no difference in the hyperglycæmia produced. If such a hyperglycæmia was caused by acidosis, one would expect it to be absent, or much reduced, in the animals previously treated with 5 .ml. isotonic sodium bicarbonate, certainly enough to mask any burn acidosis. It is thus concluded that acidosis is not a major factor in the production of rat burn hyperglycæmia.

It will be noticed that the blood sugar observed one hour after burning was less in those animals that had been given 5 ml. isotonic fluid intraperitoneally than in similar animals that had received no such treatment.

6. *Is Burn Hyperglycæmia Beneficial?*—It might be argued that the hyperglycæmia response to burning is an attempt on the part of the animal to combat the crisis associated with the period of shock. If this was so, well-fed animals, in virtue of their greater carbohydrate reserve, should be able to tolerate burning more readily than starved animals, with their depleted stores of carbohydrate. Accordingly, 24 hour-starved rats, and similar animals fed 600 mg. glucose/100 g. body-weight by stomach tube, were burned at different temperatures. Table XIV of the Appendix shows that the starved rats, with an initial blood sugar of 69 mg./100 ml. (Table VII, controls), survived the burning just as well as the fed animals, with an initial blood sugar of 104 mg./100 ml. (Table VIII, controls). It thus seems that a high blood sugar plays no striking rôle in the protection of rats from the ill-effects of burning.

DISCUSSION.

The following may be put forward as evidence that an adrenaline response occurs after burning :—

1. The changes in carbohydrate metabolism—rise in blood sugar, rise in blood lactic acid and fall in carcass glycogen—are similar to those observed after adrenaline injection [see Cori, 1931, for references].

2. The changes in carbohydrate metabolism are much less in adrenalectomised rats.

3. There is no demonstrable difference between the glycogenolytic power of liver brei from normal and from burned rabbits.

4. There is a fall in the ascorbic acid content of the adrenal gland of the rabbit, indicating some abnormal adrenal activity.

5. Hartmann *et al.* [1926], Riehl [1928], and Saito [1928 *b*] have demonstrated an increased adrenaline liberation after burning.

6. Much emphasis has been placed on the pathological changes seen in the adrenal gland after burning [see, for example, Nakata, 1915; Weiskotten, 1917, 1919; and Olbrycht, 1924].

But, on the other hand, the following is evidence that there is a second mechanism:—

1. As has been shown by Slocum and Lightbody [1931], and confirmed by us for a few animals, adrenalectomy does not entirely abolish the burn hyperglycæmia in rabbits. This may of course be due to accessory adrenal tissue not removed at operation.

2. After burning, there is a fall in liver glycogen in the rabbit and no change in the rat. At a similar time after an adrenaline injection, there is a rise in liver glycogen [see Cori, 1931, for references].

3. Liver slices from burned rabbits do not readily form glycogen from glucose; this is not so after an adrenaline injection which produces a similar rise in blood sugar. Since the metabolism of liver tissue has returned to normal within 24 hours of burning, it is highly probable that this mechanism is part of a shock reaction [Clark and Rossiter, 1943].

We are thus forced to the conclusion that there are two mechanisms contributing to the high blood sugar which follows burning. As will be seen below, this appears to be true for other types of hyperglycæmia.

The Hyperglycæmic Response.

Hyperglycæmia has been observed in patients [Cannon, 1923] and in experimental animals [Aub and Wu, 1920] suffering from traumatic shock. It also occurs during asphyxia [Kellaway, 1919], after hæmorrhage [Schenk, 1894; Rose, 1903; Anderson, 1908; Robertson, 1935; and Govier and Greer, 1941], and during many other forms of anhydræmia [Marriott, 1923], including intestinal obstruction [Draper, 1917; and Carlson and Orr, 1934] and cholera [Loh and Tai, 1936]. Also, a high blood lactic acid has been reported during shock [Macleod, 1921, and Gutmann *et al.*, 1941], during anhydræmia [Marriott, 1923], after hæmorrhage [Govier and Greer, 1941], and in cholera [Liu *et al.*, 1933], while a decrease in liver glycogen has been found during shock [Stolfi, 1936]; glycosuria has also been reported in patients suffering from shock [Thannhauser, 1916]. In some of these conditions an adrenaline liberation has been demonstrated. This is true for asphyxia [Gley and Quinquand, 1917; Cannon and Hoskins, 1911, 1919; and Kodama, 1924], shock [Bedford, 1917; and Rapport, 1922], and after

hæmorrhage ·[Bedford, 1917; Tournade and Chabrol, 1925; Saito, 1928 *a*; and Saito *et al.*, 1928]. Splanchnectomy or adrenalectomy may prevent the demonstrable liberation of adrenaline, but it does not completely abolish the hyperglycæmia [see Nishi, 1909; Tachi, 1928; and Brooks, 1935, for hæmorrhage; and Kellaway, 1919; Olmsted, 1925; and Sato *et al.*, 1932, for asphyxia], suggesting that there are two processes at work—(*a*) an adrenaline response, and (*b*) a mechanism which is active whether the adrenals are present or not.

Of all these conditions, the one that appears to have received the most study is the hæmorrhage hyperglycæmia. It has been shown, for instance, that the rise in blood sugar is less if the animals have previously been deprived of food, and that if the hepatic vessels are ligated there is not a rise, but a fall, in blood sugar after bleeding [Schenk, 1894, and Hirsch, 1915]. This indicates that the immediate source of carbohydrate is the liver, a point well illustrated by the experiments of Robertson [1935], who found a large increase in sugar in the hepatic vein after bleeding. In starved animals, hepatic stores cannot possibly account for the high rise in blood sugar, some of which must come from the muscles, as has been found by Tachi [1926].

It has now been shown that burn hyperglycæmia approximates very closely to hæmorrhage hyperglycæmia; in both conditions there is an adrenaline liberation and also a further mechanism; in both conditions the hyperglycæmia is greater in well-fed than in starved animals, and the final source of the carbohydrate is the muscles, although the immediate source is, in all probability, the liver. In addition, it has been demonstrated that the depletion of stores of muscle glycogen is accompanied by a rise of blood lactic acid. It is assumed that the mechanism is one involving the Cori cycle; muscle glycogen is mobilised in the form of lactic acid, which, in the liver, is transformed, possibly by way of glycogen, to glucose.

Acidosis.

Although there is probably a slight degree of acidosis in patients and experimental animals after burning, and acidosis of itself may produce changes in carbohydrate metabolism [Elias, 1912; Satake, 1926; Syozi, 1937; Donnelly, 1938; and Guest and Rawson, 1941], it is considered that acidosis plays little part in the production of the burn hyperglycæmia.

Practical Applications.

It should be noted that, after burning, the carbohydrate stores (liver and muscles) quickly become exhausted. Although some evidence is presented to show that carbohydrate feeding does not lessen the mortality among experimentally burned animals, it is equally true that saturating the stores with carbohydrate does no harm. It is suggested

that during the initial period of hæmoconcentration, when there is likely
to be functional impairment of the kidney together with nitrogen
retention [see Clark, Peters, and Rossiter, 1943], diets rich in carbo-
hydrate should be given.   There is also evidence that this carbohydrate
will spare protein, a point of importance when one considers the large
negative nitrogen balance which very soon occurs after severe burns
[see Cuthbertson et al., 1939; Clark, Peters, and Rossiter, 1943; and
Taylor et al., 1943].

SUMMARY.

1. Burning caused a rise in the blood sugar of both rats and rabbits
anæsthetised with ether.   In rabbits, this rise was greater in well-fed
animals than in those starved for 24 hours, and had completely dis-
appeared 24 hours after burning.   In both species, adrenaline injections
produced a similar hyperglycæmia.   In rats, whose adrenals had been
removed, there was no rise in blood sugar after burning.

2. Rats had a higher blood lactic acid one hour, but not 3 hours,
after burning; adrenaline injections produced similar changes in blood
lactic acid.

3. Both one and 3 hours after burning, there was a fall in the glycogen
content of the whole carcass (chiefly muscle glycogen) of rats.   Similar
changes were observed after adrenaline injections.   The carcass glycogen
of burned adrenalectomised animals remained normal.

4. Rabbits deprived of food for 24 hours and burned, had a lower
liver glycogen, both 4 and 24 hours after burning, than the controls,
which had been merely anæsthetised.   On the other hand, burning
produced no change in the liver glycogen of either normal or adrenal-
ectomised rats starved for 24 hours, but the injection of adrenaline
caused a slight rise.

5. Liver slices from rabbits killed 4 hours after burning formed
glycogen from glucose less readily than slices from anæsthetised control
animals.   Slices from similar animals, killed 2–4 hours after an injection
of adrenaline which produced a similar degree of hyperglycæmia, formed
glycogen normally.

6. Liver brei from burned rabbits did not break down glycogen,
either by phosphorylase or amylase activity, any more rapidly than a
similar brei from control animals.

7. There was no increase in the amylase activity of serum of rabbits
either 4 or 24 hours after burning.

8. There was a fall in the ascorbic acid content of the adrenal gland
of rabbits, both 4 and 24 hours after burning, but no change in the
ascorbic acid of the liver.

9. Injection of 5 ml. of isotonic ammonium chloride intraperitoneally
into rats caused a rise in blood sugar of the same order as that observed
after burning, but the plasma alkali reserve, after such an injection,

was much less than that observed after burning. Intraperitoneal injection of 5 ml. of isotonic sodium bicarbonate did not influence the burn hyperglycæmia any more than a similar injection of 5 ml. isotonic sodium chloride. It is concluded that acidosis plays no part in burn hyperglycæmia.

10. Well-fed rats were no better able to survive burning than similar animals starved for 24 hours.

11. The mechanism of burn hyperglycæmia is discussed. It is concluded that there are at least two distinct processes at work:

(a) the liberation of adrenaline from the adrenal glands;
(b) some other process or processes, either stimulating hepatic glycogenolysis or inhibiting glycogenesis.

12. The source of the glucose is chiefly the muscle glycogen, and it is suggested that it is mobilised by a mechanism similar to the Cori cycle.

13. As a practical measure, it is suggested that during the period of shock and hæmoconcentration burn patients should be given diets rich in carbohydrate.

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APPENDIX.

TABLE I.—BLOOD SUGAR OF RABBITS.

Previous treatment.	No. animals.	Mean blood sugar (mg./100 ml.).			S.E. mean.	P.*
		Before burning.	After burning.	Rise.		
A. Well-fed Animals.						
Control (anæsthetised only). Sample 4 hours later.	2	120	113	-7	$\pm$ 8	
Burned (30 seconds at 70° C.). Sample 4 hours later.	3	125	424	*299*	$\pm$ 56	< 0.01
B. Animals starved 24 hours.						
Control (anæsthetised only). Sample 4 hours later.	8	102	115	13	$\pm$ 9	
Burned (30 seconds at 70° C.). Sample 4 hours later.	8	109	227	*118*	$\pm$ 20	< 0.01
Burned (30 seconds at 70° C.). Sample 24 hours later.	3	115	111	-4	$\pm$ 4	0.34

* Value of P in test of significance of difference between mean rise of blood sugar of control and experimental groups.

TABLE II.—SYNTHESIS OF GLYCOGEN FROM GLUCOSE BY RABBIT LIVER SLICES. RABBIT STARVED 24 HOURS BEFORE BEING KILLED. GLUCOSE CONCENTRATION 0·5 PER CENT. BICARBONATE BUFFER. ATMOSPHERE, 95 PER CENT. O_2, 5 PER CENT. CO_2. INCUBATION TIME, 1 HOUR.

Previous treatment.	No. animals.	Mean glycogen (mg./g.).			P.
		Before incubation.	After incubation.	Synthesised (± S.E. mean).	
Control (anæsthetised only). Killed 4 hours later.	5	4·7	8·2	3·5 ± 0·2	
Burned (70° C. for 30 seconds). Killed 4 hours later.	7	1·2	1·9	0·7 ± 0·2	< 0·01
Adrenaline injected (0·3 mg./kg.). Killed 2–4 hours later.	4	3·1	6·7	3·6 ± 0·5	0·86

TABLE III.—GLYCOGEN (MG./G.) CONTENT OF LIVERS OF RABBITS STARVED 24 HOURS.

Previous treatment.	No. animals.	Glycogen (mg./g.).	S.E. mean.	Fall.	P.*
Control (anæsthetised only). Killed 4 hours later.	7	13·4	± 3·4		
Burned (30 seconds at 70° C.). Killed 4 hours later.	9	2·0	± 1·9	11·4	< 0·01
Burned (30 seconds at 70° C.). Killed 24 hours later.	4	2·0	± 0·6	11·4	0·04

* In this and subsequent tables the value of P given is that obtained in test of significance between difference of means of control and experimental groups.

TABLE IV.—GLYCOGENOLYSIS IN RABBIT LIVER BREI. FOR DETAILS SEE TEXT.

Previous treatment.	No. animals.	Phosphorolysis.				No. animals.	Amylolysis.			
		Phosphorus uptake (mg./g. wet weight, S.E. mean).					Glycogen destroyed by amylase activity (mg./g. wet weight, S.E. mean).			
		10′.	30′.	60′.	120′.		10′.	30′.	60′.	120′.
Control (anæsthetised only). Killed 4 hours later.	5	0·46	1·09 (±0·12)	1·13	0·85	4	2·4	5·8	8·7 (±1·5)	13·6
Burned (30 seconds at 70° C.). Killed 4 hours later.	6	0·80	1·25 (±0·18)	1·40	1·28	5	5·2	6·2	10·4 (±1·7)	11·3

TABLE V.—AMYLASE ACTIVITY IN SERUM OF RABBITS STARVED FOR 24 HOURS. FOR DETAILS SEE TEXT.

Previous treatment.	No. animals.	Mg. maltose formed.	S.E. mean.	P.
Control (anæsthetised only). Sample taken 4 hours later.	4	1·60	± 0·12	
Burned (30 seconds at 70° C.). Sample taken 4 hours later.	2	1·12	± 0·13	0·06
Burned (30 seconds at 70° C.). Sample taken 24 hours later.	4	1·21	± 0·16	0·09

TABLE VI.—VITAMIN C (MG./G.) CONTENT OF ADRENAL GLAND AND LIVER OF RABBITS STARVED FOR 24 HOURS.

Previous treatment.	Adrenal gland.					Liver.				
	No. animals.	Vitamin C (mg./g.).	S.E. mean.	Fall.	P.	No. animals.	Vitamin C (mg./g.).	S.E. mean.	Fall.	P.
Control (anæsthetised only). Killed 4 hours later.	10	2·9	± 0·3	..	..	8	0·12	± 0·01		
Burned (30 seconds at 70° C.). Killed 4 hours later.	13	1·9	± 0·1	1·0	< 0·01	11	0·13	± 0·01	− 0·01	0·55
Burned (30 seconds at 70° C.). Killed 24 hours later.	4	1·6	± 0·3	1·3	0·05	4	0·16	± 0·04	− 0·04	0·77

Table VII.—Blood Sugar (mg./100 ml.) of Rats Starved for 24 Hours.

Previous treatment.	No. animals.	Mean blood sugar.	S.E. mean.	Rise (mg./ 100 ml.).	P.
Control (no treatment)	14	69	±2		
Burned (30 seconds at 80° C.). Sample 1 hour later.	10	94	±5	25	< 0·01
Burned (as above). Sample 3 hours later.	9	81	±5	12	0·01
Anæsthetised only. Sample 1 hour later.	11	68	±4	− 1	< 0·9
Anæsthetised only. Sample 3 hours later.	9	72	±2	3	0·75
Adrenaline injection (0·05–0·1 mg./kg.). Sample 1 hour later.	4	105	±3	36	< 0·01
Adrenaline injection (0·05–0·1 mg./kg.). Sample 3 hours later.	4	91	±11	22	< 0·01
Adrenaline injection (0·2–0·5 mg./kg.). Sample 1 hour later.	6	141	±16	72	< 0·01
Adrenaline injection (0·2–0·5 mg./kg.). Sample 3 hours later.	9	106	±4	37	< 0·01
Adrenalectomised and burned (as above). Sample 1 hour later.	6	68	±4	− 1	0·80
Adrenalectomised and burned (as above). Sample 3 hours later.	4	70	± 4	1	0·79
Adrenalectomised only. Sample 1 hour later.	3	71	±2	2	0·63
Adrenalectomised only. Sample 3 hours later.	3	59	±4	− 10	0·04

TABLE VIII.—BLOOD SUGAR (MG./100 ML.) of WELL-FED RATS (STARVED FOR 24 HOURS AND THEN FED 600 MG. GLUCOSE/100 G. BODY-WEIGHT BY STOMACH TUBE).

Control. Determinations made 3 hours after feeding glucose.	Burned 3 hours after feeding glucose. Determinations made 1 hour later.	Burned 3 hours after feeding glucose. Determinations made 3 hours later.	Anæsthetised 3 hours after feeding glucose. Determinations made 1 hour later.	Anæsthetised 3 hours after feeding glucose. Determinations made 3 hours later.
99	108	96	103	90
111	129	110	101	96
103	120	101	98	96
Mean 104	119	102	101	94

TABLE IX.—BLOOD LACTIC ACID (MG./100 ML.) OF RATS STARVED FOR 24 HOURS.

Previous treatment.	No. animals.	Mean blood lactic acid.	S.E. mean.	Rise (mg./100 ml.).	P.
Control (no treatment) . .	9	19	± 3		
Burned (30 seconds a t80° C.). Sample 1 hour later.	8	35	± 5	16	< 0·01
Burned (as above). Sample 3 hours later.	8	14	± 1	− 5	0·11
Anæsthetised only. Sample 1 hour later.	9	18	± 3	− 1	0·77
Anæsthetised only. Sample 3 hours later.	6	13	± 2	− 6	0·12
Adrenaline injection (0·05–1 mg./kg.). Sample 1 hour later.	4	38	± 3	19	< 0·01
Adrenaline injection (0·05–1 mg./kg.). Sample 3 hours later.	4	23	± 2	4	0·36
Adrenaline injection (0·2–0·5 mg./kg.). Sample 1 hour later.	4	47	± 9	28	< 0·01
Adrenaline injection (0·2–0·5 mg./kg.). Sample 3 hours later.	8	25	± 2	6	0·10

TABLE X.—CARCASS GLYCOGEN (MG./100 G. CARCASS WEIGHT) OF RATS STARVED FOR 24 HOURS.

Previous treatment.	No. animals.	Mean carcass glycogen.	S.E. mean.	Fall (mg./ 100 g.).	P.
Control (no treatment) . .	10	245	± 14		
Burned (30 seconds at 80° C.). Killed 1 hour later.	6	178	± 11	67	< 0·01
Burned (as above). Killed 3 hours later.	6	145	± 10	100	< 0·01
Anæsthetised only. Killed 1 hour later.	6	225	± 13	20	0·35
Anæsthetised only. Killed 3 hours later.	6	230	± 10	15	0·46
Adrenaline injection (0·01–0·2 mg./kg.). Killed 1 hour later.	4	177	± 6	68	0·01
Adrenaline injection (0·01–0·2 mg./kg.). Killed 3 hours later.	4	143	± 6	102	< 0·01
Adrenaline injection (0·1–0·2 mg./kg.). Killed 1 hour later.	5	158	± 14	87	< 0·01
Adrenaline injection (0·1–0·2 mg./kg.). Killed 3 hours later.	9	147	± 6	98	< 0·01
Adrenalectomised and burned (as above). Killed 1 hour later.	6	200	± 6	45	0·03
Adrenalectomised and burned (as above). Killed 3 hours later.	4	235	± 19	10	0·69
Adrenalectomised only. Killed 1 hour later.	3	246	± 19	– 1	0·9
Adrenalectomised only. Killed 3 hours later.	3	205	± 21	40	0·18

TABLE XI.—LIVER GLYCOGEN (MG./100 G. CARCASS WEIGHT) OF RATS
STARVED FOR 24 HOURS.

Previous treatment.	No. animals.	Mean liver glycogen.	S.E. mean.	Rise (mg./ 100 g.).	P.
Control (no treatment) . .	10	6·3	± 0·2		
Burned (30 seconds at 80° C.). Killed 1 hour later.	5	5·8	± 0·6	− 0·5	0·39
Burned (as above). Killed 3 hours later.	7	6·3	± 0·2	0	
Anæsthetised only. Killed 1 hour later.	7	7·2	± 0·4	0·9	0·07
Anæsthetised only. Killed 3 hours later.	7	6·8	± 0·3	0·5	0·22
Adrenaline injection (0·05–0·1 mg./kg.). Killed 1 hour later.	3	6·0	± 0·6	− 0·3	0·59
Adrenaline injection (0·05–0·1 mg./kg.). Killed 3 hours later.	4	7·3	± 0·5	1·0	0·07
Adrenaline injection (0·2–0·5 mg./kg.). Killed 1 hour later.	5	6·9	± 0·4	0·6	0·19
Adrenaline injection (0·2–0·5 mg./kg.). Killed 3 hours later.	9	8·2	± 0·9	*1·9*	*0·05*
Adrenalectomised and burned (as above). Killed 1 hour later.	6	6·2	± 0·3	− 0·1	0·81
Adrenalectomised and burned (as above). Killed 3 hours later.	4	6·6	± 0·4	0·3	0·52
Adrenalectomised only. Killed 1 hour later.	2	5·9	± 0·8	− 0·4	0·55
Adrenalectomised only. Killed 3 hours later.	3	5·7	± 0·3	− 0·6	0·24

TABLE XII.—BLOOD SUGAR AND PLASMA ALKALI RESERVE OF RATS STARVED FOR 24 HOURS.

Previous treatment.	Blood sugar (mg./100 ml.).					Alkali reserve (ml. CO$_2$/100 ml.).				
	No. animals.	Blood sugar.	S.E. mean.	Rise.	P.	No. animals.	Alkali reserve.	S.E. mean.	Fall.	P.
Control (anæsthetised only).	11	69	± 4	..	..	8	50·3	± 1·1		
Burned (30 seconds at 80° C.). Sample taken 1–1½ hours later.	11	83 *	± 4	14	0·03	7	48·7	± 2·1	1·6	0·5
Injected with 50 c.c. isotonic ammonium chloride. Sample taken 1–1½ hours later.	8	89	± 6	20	0·02	7	32·2	± 1·8	18·1	< 0·01

* It will be noticed that whereas the control blood sugar is the same, the blood sugar after burning is less than that reported in Table VII. The rats used in this experiment were of a different strain.

TABLE XIII.—BLOOD SUGAR OF RATS STARVED FOR 24 HOURS AND THEN BURNED FOR 30 SECONDS AT 80° C. ANIMALS KILLED 1 HOUR AFTER BURNING.

Previous treatment.	No. animals.	Blood sugar (mg./100 ml.).	S.E. mean.	P.
Injected with 5 ml. isotonic sodium chloride.	6	77·0	± 6·2	
Injected with 5 ml. isotonic sodium bicarbonate.	6	76·5	± 5·7	>0·9

TABLE XIV.—MORTALITY OF RATS STARVED FOR 24 HOURS AND THEN
BURNED FOR 30 SECONDS AT THE STATED TEMPERATURE.

Temperature of burning.	Fed 600 mg. glucose in 2 ml. water/100 g. body-weight by stomach tube.		Fed 2 ml. water/100 g. body-weight by stomach tube.	
	No. animals.	No. dead in 24 hours.	No. animals.	No. dead in 24 hours.
70°	6	1	6	0
80°	6	1	6	1
85°	6	1	6	0
90°	6	5	6	5

SUBCUTANEOUS TEMPERATURES IN MODERATE TEMPERA-TURE BURNS.[1] By K. Mendelssohn and R. J. Rossiter. From the Department of Biochemistry, Oxford.

The macroscopic and microscopic damage caused by the application of a burning iron of moderate temperature to the skin of guinea-pigs has been previously reported [Leach, Peters, and Rossiter, 1943]. An application for 1 minute at 45° C. causes no macroscopic and negligible microscopic damage. At 50° C. there is a temporary erythema and the histological damage—swelling of nuclei of epithelial cells and loss of basophil staining of their cytoplasm—is completely reversible. At 55° C., following a transient blanching, there is a persistent erythema, flare, slight œdema, histological evidence of separation of the prickle cells from the basement membrane, disintegration of some of these cells, greater loss of basophil properties and escape of pyronin-staining substance from the cytoplasm of the epithelial cells, fragmentation of their nuclei and the appearance of Feulgen-positive material (nucleo-protein) in the incipient blister spaces; the damage is irreversible and, later, a firm scab forms, which subsequently is shed. At 60° C. the erythema, flare, and œdema is more pronounced and the epidermis can be readily separated from the dermis at the basal cell layer; micro-scopically, the epithelial cells are heat coagulated; microscopically, there is gross damage to epithelial cells and also changes in the collagen fibres—swelling, partial disintegration, increase in affinity for basic dyes and decrease for acidic.

All the temperatures, so far referred to, are those of the burning iron. It is obvious that the temperature of the tissues must be lower. The experiments reported below were designed with the object of defining more accurately the temperature at which a known degree of damage is produced.

We have been able to find but few references in the literature to the recording of subcutaneous temperatures during local application of heat and, of these, most refer to man. Grunspan [1913] reported that 15 minutes diathermy produced pain with a subcutaneous temperature

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—measured by an iron-constantan thermocouple—of 40·5°. Hot air blown on the skin caused much pain, and even second degree burns, with no significant rise in the subcutaneous temperature. Lewis and Love [1926], using a copper-constantan thermocouple, found that, when a heated copper bar was placed on human skin, the limit of tolerance was 47–48·5° C., corresponding to a subcutaneous temperature of 42–43° C. If, on the other hand, a limb were immersed in warm water, the tolerance limit was 45–47° C. [Lewis, 1924, and confirmed by us]. At 43–44° C., with a subcutaneous temperature of 37–40° C., there was a flushing of the skin, and 51–52° C. (subcutaneous temperature of 42° C.) produced blistering and wheal formation. Westermark [1927] heated the skin of animals with high frequency currents and recorded the intracutaneous temperature. Rats tolerated 45° C. for $1\frac{1}{2}$ hours and 46° C. for 1 hour, but 46° C. for $1\frac{1}{2}$ hours gave "obvious necrosis." For the pig (? guinea-pig), 50° C. for 4 minutes was without effect, while 50° C. for 8 minutes produced necrosis. Details of these experiments are very meagre.

Animals.—These were guinea-pigs of weight 500–700 g.

Method of Burning.—Burns were produced by means of the burning iron previously described by Leach *et al.* [1943].

Method of Recording Temperature.—The temperature was measured with a copper-constantan thermocouple inserted in the tip of a hypo-dermic syringe needle of 0·45 mm. outside diameter. The reference junction was thermally connected to a large block of copper, whose temperature could be maintained at an even level by means of an electric heater. The resultant thermo-e.m.f. was read on a simple Cambridge Instrument Pot Galvanometer of 45 ohms resistance, the scale being 100 cm. from the mirror of the instrument. The accuracy thus obtained was 0·05° C.

In order to determine whether an appreciable error was introduced by thermal conduction along the needle, its shaft was packed with solid CO_2 at a distance of 2·5 cm. from the tip while the latter was in the recording position. This artificially created temperature difference of more than 100° C. influenced the reading by only 0·05° C. Since the largest temperature difference encountered in the experiments was only about 35° C., it is clear that any error due to heat conduction was negligible.

The needle was inserted through the skin of the shaved anæsthetised guinea-pig and moved into the loose subcutaneous tissue. It was then directed upwards so that the point was in contact with the lower surface of the dermis. While the burning was in progress, the pressure on the iron depressed the skin firmly against the point of the needle; the temperature measured, therefore, was that of the deepest layer of the dermis. The epithelial cells were at a higher temperature, a temperature intermediate between that of the burning iron and that recorded. Some

experiments were performed with the thermometer needle intradermally. These results were not readily reproducible, however, possibly because of the difficulty of accurately localizing the point of the needle, and possibly also because of the seepage of œdema fluid along the path of damaged tissue, produced when the needle was inserted.

Anæsthesia.—At the outset, ether was used as an anæsthetic, but it soon became evident that the skin temperature fell rapidly—4–5° C. per hour—when this substance was employed. Fig. 1 shows that this fall was due to a fall in body temperature rather than to changes in the
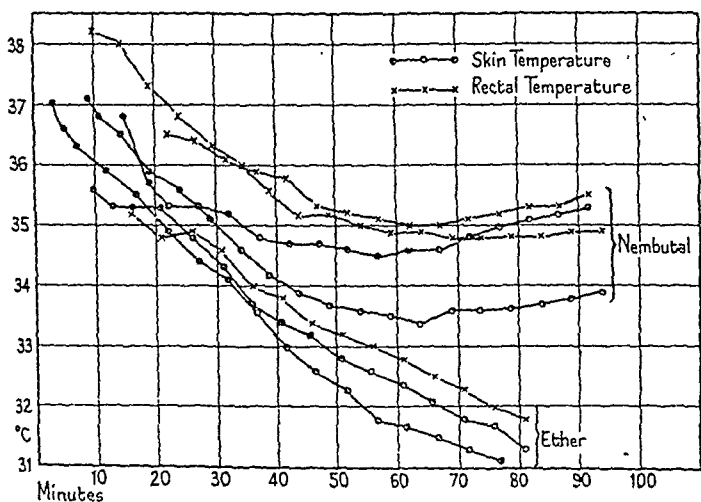
Fig. 1.—Subcutaneous and rectal temperatures of guinea-pigs under either ether or nembutal anæsthesia.

cutaneous blood supply. Under these conditions, the changes in the initial skin temperature (5° or so in the same animal) seriously influenced the final temperature reached; this was particularly so for 1-minute applications of the burning iron. It was found, however, that intraperitoneal nembutal (40 mg. kgm.) gave the same degree of narcosis— abolition of corneal reflex but retention of pinna reflex to high-pitched squeak—but a much smaller fall in temperature (fig. 1) once the required depth of anæsthesia had been reached. After an initial drop, usually lasting 40 minutes, a steady temperature persisted for an hour or more. Readings were taken during this period. Reference to the literature has shown that the fall in body temperature of experimental animals during ether anæsthesia is well known [Allen, 1896; Mills, 1907; and Davis, 1909].

Horizontal Distribution of Temperature under Burning Iron.

The horizontal distribution of the subcutaneous temperature was determined in a number of experiments with the burning iron at 55° C.

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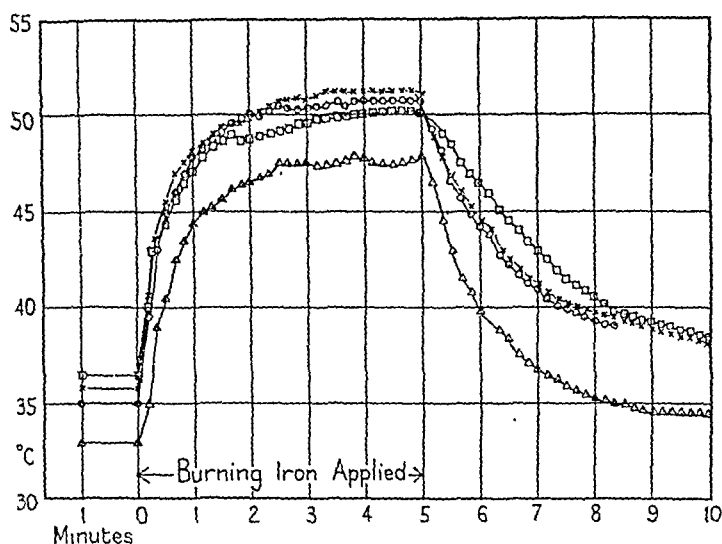
Fig. 3.—Time distribution of subcutaneous temperature under the burning iron. Temperature of iron, 55° C. Guinea-pig under nembutal anæsthesia.
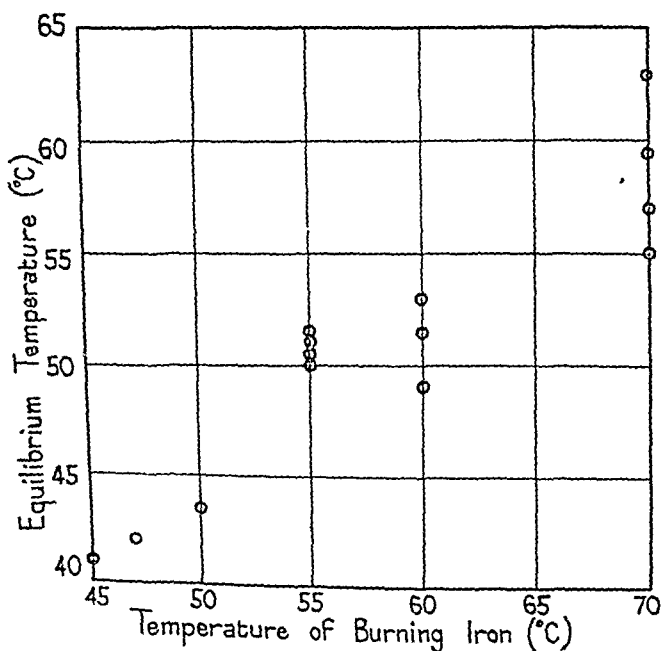

Fig. 4.—Equilibrium subcutaneous temperature under the burning iron. Guinea-pig under nembutal anæsthesia.

The iron was placed on the skin until equilibrium had been attained and then the point of the thermometer needle was drawn under the iron in a series of steps, 3 mm. at a time, the equilibrium temperature being recorded before each movement. Fig. 2 shows the subcutaneous temperature under the iron. Such a temperature gradient had been predicted from a knowledge of the horizontal distribution of histological damage reported previously [Leach *et al.*, 1943]. This also shows that
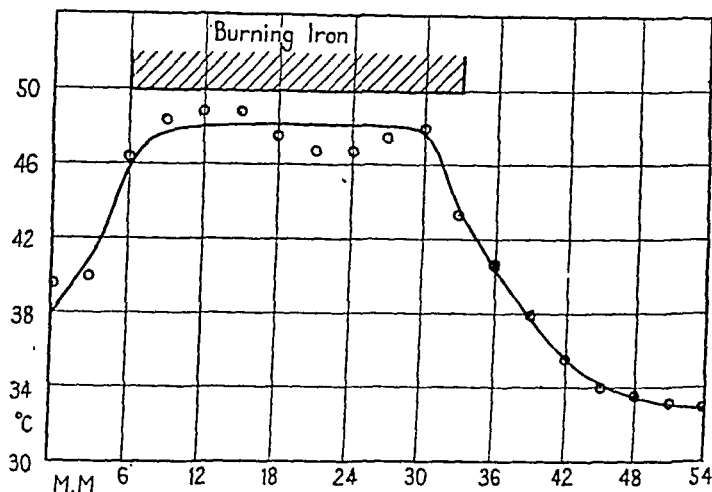
Fig. 2.—Horizontal distribution of subcutaneous temperature under the burning iron. Temperature of iron, 55° C. Guinea-pig under nembutal anæsthesia.

a slight accidental displacement of the needle from a position directly beneath the centre of the iron is unlikely to falsify appreciably the recorded temperature.

Equilibrium Temperatures.

After applying the burning iron to the skin, the subcutaneous temperature was recorded at 10-second intervals until equilibrium had been reached. Fig. 3 shows the curve obtained for four different animals with the burning iron at 55° C. It also gives some idea of the degree of reproducibility which may be expected. Similar curves were plotted for temperatures of the burning iron varying from 45° to 70° C. Fig. 4 shows the relationship between the equilibrium temperature and the temperature of the burning iron. With a burning-iron temperature of 55–60° C., corresponding to an equilibrium temperature in the neighbourhood of 50° C., there is a flattening of the curve. It would be interesting to know the cause of this irregularity. It is possibly due to some physiological phenomenon producing a change in the rate of heat removal by blood-stream convection. Another possibility is that the thermal properties of the skin are changed, for it is at these temperatures that

TABLE I.—TEMPERATURE REACHED AFTER A 1-MINUTE APPLICATION OF THE BURNING IRON TO THE SKIN OF ANÆSTHETISED GUINEA-PIGS.

Temperature of burning iron.	No. of observations.	Mean temperature reached.	Standard error of mean.	P.
45	9	38·4	0·2	< 0·01
47	8	40·1	0·4	< 0·214
50	11	41·1	0·6	< 0·01
55	16	44·8	0·6	< 0·01
60	14	48·4	1·0	< 0·01
70	7	54·4	1·1	< 0·01

DISCUSSION.

These findings enable us to state to within what limits of temperature the *cells* of the skin must be subjected, to produce certain of the changes previously observed. This temperature must be intermediate between that of the burning iron and that of the under surface of the skin. Erythema is produced when the cells of the skin are heated to some temperature between 40° and 50° C. Irreversible destruction, characterized by persistent erythema, flare, slight œdema, scabbing, loss of basophil properties and escape of a pyronin-staining substance from the cytoplasm of the epithelial cells and of nucleoprotein from their nuclei, occurs at temperatures between 45° and 55° C. Massive œdema, separation of the epidermis and coagulation of the epithelium occurs between 50° and 60° C. That irreversible damage is sustained (*i.e.* cells are killed) between 45° and 50° C. brings skin tissue into line with the observations of other workers on the ability of living tissues generally to withstand heat [Leach *et al.*, 1943, for references].

An important feature of this study is that it forms a basis for the correlation of "*in vitro*" enzyme studies, now proceeding, with the conditions actually occurring in the heat-damaged epithelial cell.

SUMMARY.

1. For a corresponding degree of narcosis, nembutal caused a lesser fall in the body temperature of guinea-pigs than ether.

2. The horizontal distribution of subcutaneous temperature under the previously described burning iron has been plotted.

3. For temperatures of the burning iron ranging from 45° to 70° C., observations have been made of the final equilibrium subcutaneous temperature; similar observations have also been made on subcutaneous temperature reached after a 1-minute application of the iron.

there is a sudden increase—of the order of 100 per cent.—in water content of skin [Leach *et al.*, 1943].

Burns for One Minute.

In previous work from this laboratory on moderate temperature burns, 1 minute has been adopted as the standard burning time. It was, therefore, of interest to determine the highest subcutaneous temperatures that would be reached during a 1-minute application, with the burning iron at different temperatures. A glance at fig. 3 shows that, whereas for a given burning-iron temperature the final equilibrium temperature reached was usually the same,[1] the temperature reached after an application of 60 seconds often varied considerably. This contrast between equilibrium and 1-minute temperatures was possibly due to different rates of heating in different animals. The temperature reached after a 1-minute application to a number of different animals is shown in fig. 5. In general, the higher the initial skin temperature the higher
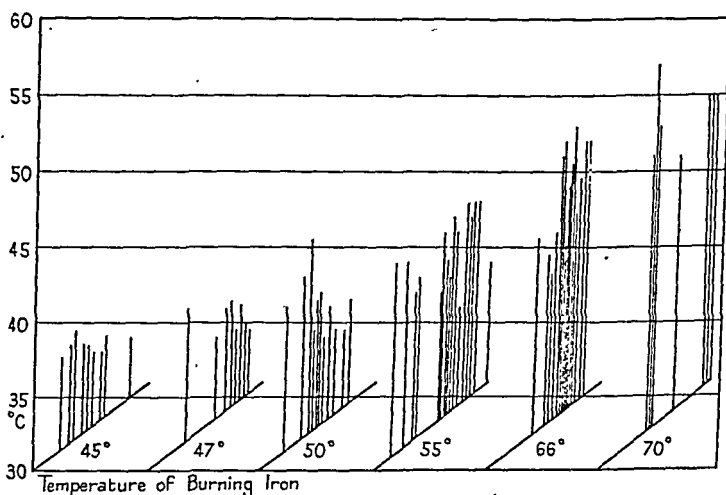
Fig. 5.—Initial and final subcutaneous temperature for a 1-minute application of the burning iron. Guinea-pig under nembutal anæsthesia.

was the temperature reached. Table I gives the mean values of the subcutaneous temperatures for each burning-iron temperature, together with the standard error of the mean. The significance of the difference between each pair of means is illustrated by the values of P also tabulated.

[1] This was certainly true for the lower burning-iron temperatures. When the temperature was higher there was a certain amount of scatter (see fig. 4).

THE INFLUENCE OF METABOLIC STIMULANTS ON WOUND HEALING; THE INFLUENCE OF THYROID and 2-4-α DINITROPHENOL. By T. H. C. Barclay,[1] D. P. Cuthbertson, and A. Isaacs, Institute of Physiology, University of Glasgow.

The daily subcutaneous injection of an alkaline extract of the anterior lobe of ox pituitary gland into the rat has no appreciable effect on the rate of healing of experimental wounds, in spite of its definite anabolic effect [Cuthbertson, Shaw, and Young, 1941]. Davidson and Waymouth [1943] found that similar extracts, which had shown growth accelerating activity in young rats, did not show a growth-promoting effect on chick heart fibroplast cultures.

Since this extract of the anterior pituitary gland had no significant action on the rate of wound healing, the influence of catabolic stimulants (e.g. thyroid gland extract and 2-4-α dinitrophenol) on the rate of wound healing was investigated. The results are recorded below.

Lauber [1930] has drawn attention to the value of thyroid administration clinically in wound healing. Kosdoba [1934], as the result of a study of incised wounds made on a variety of species, has come to the conclusion that, as judged by macroscopic and microscopic appearances, the healing times are definitely reduced in all species by the injection of thyroxin or by homo- or hetero-transplantation of thyroid gland, and that this takes place in wounds healing both by first and second intention. Further, he found that partial (up to $\frac{3}{4}$) resection of the thyroid or total thyroidectomy delayed healing by 4–40 days.

EXPERIMENTAL.

The technique of wounding and measurement of healing was that previously described by Cuthbertson et al. [1941]. This involves the removal of discs of skin from the outer aspects of the thighs. The criterion of healing was based on the complete epithelialisation of the wounded area (macroscopic observation) with subsequent confirmation by histological methods. By the latter confirmatory test it was possible to demonstrate that the healing process was shared by the whole thick-

[1] James Reid Stewart post-graduate Bursar.

309

4. The temperatures, at which the changes produced by burning occur, have been defined with greater accuracy.

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lost weight.   In Expt. 2 the loss was less in the thyroid (2) group than in thyroid (1) group.

Expt. 4.—In this experiment the 2–4-*a* dinitrophenol was administered orally, beginning on the day of operation.

The diet for this group was made up as follows: to 0·12 g. 2–4-*a* dinitrophenol N/NaOH was added until the solid dissolved and the solution was just alkaline, using phenolphthalein as indicator.   This volume was made up to 50 ml. with ethyl alcohol and the solution added, drop by drop with constant stirring, to 1 kg. stock diet.   The alcohol was then evaporated off in an incubator room with an air current.

There were 16 wounds (8 animals) in the control group and the same number in the dinitrophenol group.   The changes in body-weight were indeterminate.

Expt. 5.—Since Murphy [1938] found that much larger doses of 2–4-*a* dinitrophenol could apparently be administered with safety to rats, the dosage in this experiment was increased approximately eight-fold, 0·9 g. 2–4-*a* dinitrophenol being added to 1 kg. of stock diet.

Originally there were 24 wounds (12 animals in each group), but 4 dinitrophenol treated animals died and 1 of the wounds of the control group was interfered with by the animal concerned.   There was a definite loss of body-weight in the dinitrophenol group.

Expt. 6.—In this experiment the dose of 2–4-*a* dinitrophenol was reduced to that of Expt. 4.   The experiment was done in combination with Expt. 4.   Originally there were 32 wounds in each group, but 3 control animals died (*i.e.* 6 wounds deficient).   All the dinitrophenol treated animals remained alive.   There was only a slight loss of body-weight in the dinitrophenol group.

EXPERIMENTAL DATA.

Since the basis of comparison between the controls and the treated animals in these experiments is the average number of days taken by the wounds to heal, the latter has been calculated for each group and is given in column 3 of the table.

As a criterion of significance the difference between the means must exceed twice the standard error of the difference between the means (in which case the odds are more than 100 to 5 against it having arisen by chance).   It will be seen from the table that the difference between the means of the controls and treated group is significant except in Expt. 5.

DISCUSSION.

Kosdoba [1934] in his experiments on the white rat noted that, so far as macroscopic appearances were concerned, the period of wound

ness of the skin and that the apparent healing on naked-eye view was not merely a covering of epithelium over incompletely healed deeper layers.

Young adult male rats were used in all the experiments. Each rat received two circular wounds each of 12·5 mm. diameter.

Throughout the experiment the animals were given, *ad libitum*, the stock diet described by Thomson [1936]. They were weighed at regular intervals in order to determine that the activity of the catabolic stimulant was being maintained.

Sepsis was observed in none of the wounds.

Experimental Notes.

Expt. 1.—The thyroid-fed group diet was made up as follows: Ext. thyroid sicc. B.P. was added in the proportion of 1 g. to 200 g. of stock food.

There were 24 wounds (12 animals) in the control group and there would have been the same number in the thyroid group, had one animal not died and one wound been chewed by its owner.

In the thyroid group (T), consumption of this diet started 8 days prior to operation and was continued throughout the experimental period.

Expt. 2.—In this experiment the proportion of ext. thyroid sicc. B.P. added to the stock diet was reduced to 0·75 g. per 200 g. With the above exception in quantity of thyroid extract, the thyroid group (1) was similar to that of Expt. 1 with regard to the pre-operative feeding. In thyroid group (2) the animals began their thyroid diet on the day of operation.

There were 24 wounds in the control group (12 animals), 18 wounds in thyroid group (1), and 22 wounds in thyroid group (2). Since the conditions of the experiment, as far as the thyroid group (1) were concerned, were the same as in Expt. 1, with the exception of dosage, it was felt that to use the animals available to the best advantage it would be preferable to have controls and the unknown thyroid group (2) as nearly as possible the same, and allow the deficiency, due to the insufficient number of animals available, to exist in thyroid group (1). Unfortunately one of the thyroid group (2) animals died, hence there were only 22 wounds to investigate.

Expt. 3.—The technique was identical with that of Expt. 2, T(1). There were 26 wounds (13 animals) in the control group. Originally the experiment had 32 wounds in each group (16 animals per group). Three rats died in the thyroid group, and the same number in the control group. One wound of the thyroid group was chewed and was in consequence discounted.

In all three experiments (1, 2, and 3) the thyroid treated groups

and Freeman [1940], have satisfactory evidence in respect of dinitrophenol. Associated with this increase in oxygen uptake is a tendency for a slight rise in general body temperature. There is also ample evidence that the thyroid gland exerts a positive influence in speeding up cardiac output, circulation time, and, in consequence, the blood supply to the part. Kosdoba [1934] has attributed the beneficial effect of this hormone to its influence on the general metabolism, but, in particular, on fibrous tissue and the blood vascular system. There is no doubt but that measures which improve the blood supply to a part, influence favourably the healing of a wound of that part [Arey, 1936].

Although Simkins [1937] has found that "dinitrophenol is remarkable for its lack of significant effects on the cardiovascular system, even when the basal metabolic rate is raised to high levels," it is difficult to conceive of an increase in the metabolic rate without some parallel change in the circulation rate. Indeed, Cushny et al. [1941] have definitely stated that dinitrophenol causes a marked increase in the pulse rate.

After the infliction of a wound, there appears to be a rapid fall in the metabolism of the tissues, the degree of fall being presumably related to the trauma. Green's [1943] and Bielschowsky and Green's [1943] recent observations on the reduction in body temperature which follows injection of muscle shock factor(s) from muscle is of special interest. There then follows a rise in metabolism above normal, followed finally by a decline to normal level [Gaza and Gissel, 1932]. One of us [Cuthbertson, 1942] has already discussed at some length the significance of this swing of the metabolic pendulum. It would appear that the action of the thyroid and dinitrophenol in enhancing the rate of wound healing may well depend on (a) a direct effect on the cells, in particular the oxidative phase of their metabolism, and which in consequence probably increases the blood supply to the part; or on (b) a stimulant action on cell proliferation, due to the products of the excess catabolism. These factors, singly or in conjunction, may prove to be the determining agents.

In these experiments, only two concentrations of dinitrophenol were tested, one 0·012 per cent., the other 0·09 per cent. With the latter dosage, the animals rapidly lost weight, and no favourable influence on wound healing was noted; indeed, rather the reverse was found. This was obviously a toxic dose. With the lower dosage no continued loss of body-weight took place. This was rather unexpected. It may have been that in proportion to the rise in metabolic rate the dinitrophenol treated animals ate more food than did the thyroid treated group. This is purely surmise since the intakes were not measured. It is of interest that despite loss in body-weight (6–17 per cent.), the thyroid treated animals exhibited a decrease in the average period of wound healing.

Experiment.	No. of wounds.	Mean period required for healing.		Difference between controls and treated.		Percentage difference of mean periods.
		Days.	± S.E. of mean.	Days.	± S.E. of diff.	
1 C	24	20·38	± 0·37	4·52	± 0·61	22%
T	21	15·86	± 0·49			
2 C	24	17·29	± 0·62			
T (1)	18	14·78	± 0·34	2·51	± 0·71	14%
T (2)	22	15·36	± 0·45	1·93	± 0·77	11%
3 C	26	18·65	± 0·61	2·25	± 0·70	12%
T	25	16·40	± 0·34			
4 C	16	21·13	± 0·53	5·69	± 0·62	27%
D.N.P.	16	15·44	± 0·31			
5 C	23	17·83	± 0·62	0·58	± 0·82	3%
D.N.P.	16	17·25	± 0·54			
6 C	26	18·88	± 0·69	2·91	± 0·75	15%
D.N.P.	32	15·97	± 0·29			

C=Control.　T=Thyroid treated.　D.N.P.=dinitrohphenol treated.

healing of his incised linear wounds was reduced from 6–8 days (controls) to 4–6 days in the injected or implanted series, a 19 per cent. reduction in time. Microscopically, the wounds were healed in 14–18 days in the injected or implanted series, a 22 per cent. reduction from the time taken to heal the controls, viz. 18–25 days. It is to be noted that Kosdoba only used 29 animals in his rat series, and of these, controls appear to have amounted to somewhere in the region of one-quarter of the total.

In the present series of experiments, thyroid gland and 2-4-a dinitrophenol have been shown to diminish significantly the mean time required for healing. The former, if given for some days before wounding, caused a reduction of 12–22 per cent. in the mean time required for healing, but when the thyroid was given from the day of wounding till healing took place then the reduction of the mean time for healing was found to be less, viz. 11 per cent. With dinitrophenol, the reductions noted in the two favourable experiments were 15 and 27 per cent. These values for the thyroid-fed animals agreed remarkably well with Kosdoba's figures for linear wounds.

Thyroid gland and dinitrophenol have the common property of increasing the rate of oxidation of the tissues. The evidence in respect of the thyroid gland is overwhelming, and Jeney and Valyi-Nagi [1938],

sidered wise to attempt to influence the rate of normally healing wounds of patients by such stimulants.

In conclusion, we wish to thank Dr. E. Lewis-Faning and Dr. P. L. McKinlay for their assistance in the statistical treatment of this investigation. We are also greatly indebted to the Medical Research Council, and to the Rankin Medical Research Fund of the University of Glasgow, for grants in aid of this work.

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It is to be noted, however, that there is some evidence to suggest that fat is the main material catabolised under the influence of dinitrophenol [Cushny et al., 1941]. Thus, there would be less change in body-weight per calorie of energy liberated than would probably occur under the influence of thyroid. Further, the end products of metabolism from the action of these two catabolites would be quantitatively and probably qualitatively different. Although not clear-cut, the evidence does suggest that the dominant factor in speeding-up the wound healing is the direct stimulant action on the cells rather than the effect of the end products of that action.

Young, Fisher, and Young [1941] found that a second wound inflicted some 10–12 days after the primary, healed at a significantly more rapid rate than the initial wound. They deduced that some accelerating factor must operate in the closure or healing of secondary wounds which is lacking in relation to primary wounds. This substance might be a "growth-promoting substance" liberated by primary wounds or a by-product of immunity, or some other principle. The simplest explanation is that in the Young et al. experiments the second wound was produced during the final phase of the enhanced metabolism which follows injury, and which has been shown to be accompanied by marked evidence of an increased catabolism and traumatic fever [Cuthbertson, 1932, 1942].

This enhanced metabolism which follows the initial phase of depressed activity would appear to be due in the first instance to substances set free from the traumatised cells. The need to label these as "active wound-healing substances" is not necessary, and tends to deflect attention from the reactive mechanism which is the essential thing.

In conclusion, the writers do not consider it wise to attempt to reduce the normal rate of wound healing in patients by such means as have been tried in these experiments. On the other hand, with indolent wounds the question of the administration of thyroid might be considered.

SUMMARY.

1. Dried thyroid gland, when fed to young adult male albino rats throughout the period of healing of circular skin wounds, caused a significant reduction in the mean time required for healing (11 per cent. reduction). When the rats were, in addition, given a pre-period of thyroid feeding before operation, the mean time required for healing was reduced by 12 and 22 per cent. in the two experiments performed.

2. 2–4-a dinitrophenol fed as a component of the diet (0·012 per cent.) also caused a significant reduction in the mean time required for healing (15 to 27 per cent. reduction). Larger doses (0·09 per cent.) lacked this stimulant effect and the weight loss was marked.

3. The significance of these findings is discussed. It is not con-

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BIOLOGICAL ASSAY AND STANDARDISATION OF MELANO-
PHORE EXPANDING PITUITARY HORMONE. By F. W.
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CONTENTS.

I. INTRODUCTION.

THE posterior lobe of the pituitary consists of two anatomically distinct structures—*pars intermedia* and *pars nervosa*. The *pars intermedia* manufactures melanophore expanding hormone and the *pars nervosa* a substance (or substances) with oxytocic, pressor, and antidiuretic activities. Unfractionated extracts of posterior lobe exhibit all four activities.

The substance(s) responsible for pressor, oxytocic, and antidiuretic effects has not been isolated in pure form. So the activity of an unknown extract is compared with that of an international standard posterior lobe powder (I.S.P.P.) prepared from ox glands according to a specified procedure and kept at the National Institute for Medical Research at Hampstead. Dale [1940] provides a fully documented review of events leading up to the adoption of this international standard. An international unit of pressor, oxytocic, or antidiuretic activity is defined as that amount of activity present in 0·5 mg. of the I.S.P.P.

There has been no formal recognition of an international unit of melanophore expanding activity, referred to as "B" (after Hogben).

TABLE II.—ASSAY TO DETERMINE NUMBER OF L.W. UNITS PER MILLIGRAM INTERNATIONAL STANDARD POWDER.

(i) Two groups, (C and D) of 6 intact *Xenopus* selected for approximately equal sensitivity and from the same group used to construct the dose-response curve (fig. 4).
(ii) Initial extractions of powder with dilute acetic acid as prescribed on p. 8 of M. 36 (1936) and British Pharmacopœa. Dilutions made with saline prescribed on p. 10.
(iii) All injections 0·25 c.c. D.L.S. All assays on white background with constant overhead illumination 15° C.

Date.	First International Standard Powder.					L.W. Standard Powder.					L.W. units per milligram International Standard Powder.
	Group of 6 toads.	Dose in gamma (·001 mg.).	Max. M.I.	Average max. M.I. from 12 toads.	"Relative" dose read from fig. 4.	Group of 6 toads.	Dose in L.W. units.	Max. M.I.	Average max. M.I. from 12 toads.	"Relative" dose read from fig. 4.	
27.5.43	C	1·25	3·5	3·25	1·3	D	1·25	3·0	3·1	1·15	$\dfrac{1·3}{1·15} \times \dfrac{1000}{1} = 1100$
28.5.43	D	1·25	3·0			C	1·25	3·3			
29.5.43	C	0·625	2·7	2·5	0·55	D	0·625	2·4	2·6	0·6	$\dfrac{0·55}{0·6} \times \dfrac{1000}{1} = 900$
30.5.43	D	0·625	2·3			C	0·625	2·8			
26.6.43	C	1·25	3·3	3·25	1·3	D	1·25	3·2	3·3	1·4	$\dfrac{1·3}{1·4} \times \dfrac{1000}{1} = 950$
27.6.43	D	1·25	3·2			C	1·25	3·4			

In previous studies on this hormone we hesitated to suggest a melano-
phore unit as the activity in 0·5 mg. of I.S.P.P., because this might
have been taken to *imply* that any powder made according to standard
procedure (or approximating to it) would contain "B" in the same
proportion to the other three activities as the international standard.
At the time we had no èvidence to show whether this was true or not.
As a temporary measure we took one of our own products of proved
stability as a standard, and defined an L.W. unit as the melanophore
activity exhibited by 0·25 microgram of it [Landgrebe and Waring,
1941]. Later work has shown two things:

(a) There is no fixed ratio of melanophore to pressor activity in
    whole extracts of different ox posterior lobe powders (Table I).
    So while it is desirable to define the melanophore unit as the
    activity in 0·5 mg. of international powder, this must not be
    taken to imply that substandards accurately assayed for
    pressor and/or oxytocic activities necessarily have a "B"
    content commensurate with that of I.S.P.P.

(b) 0·5 mg. of I.S.P.P. contains "B" activity equivalent to 500 L.W.
    units (Table II), *i.e.* 1 L.W. unit = 2 "international" milli-units.

TABLE I.—ACTIVITY RECORDED AS PERCENTAGE OF INTERNATIONAL
STANDARD POWDER.

Powder.	Pressor.	Oxytocic.	"B."
1st International Standard .	87	87	103
2nd International Standard .	100	100	100
Canadian Standard . . .	100 *	100 *	40 †
Substandard used for routine pressor assays . . .	90	80	17
Commercial (Oxo) . . .	65	50	15
,, (Duncan Flockhart)	70	70	60
F.G.Y. ‡	60	50	18

* Assumed.
† Calculated from Stehle's [1936] figures.
‡ A powder kindly supplied by Dr. F. G. Young of the M.R.C.

II. THEORETICAL CONSIDERATIONS.

When a "B"-containing extract is injected into pale test animals
(*e.g.* certain amphibia and fish) the melanophores expand and the skin
darkens. The skin then gradually gets paler as the melanophores
contract. To use this sequence of events for assay purposes we must
(a) measure melanophore expansion or darkening of the skin, (b) adopt
a criterion for estimating relative potencies of extracts. These will be
considered separately.

used for fig. 1) that an animal with few but fully expanded melanophores is paler than an animal with abundant contracted ones.

Quantitative results of real value can only be obtained by a direct estimation of the degree of expansion of melanophores themselves. Hogben, who was the first to realise this, introduced his first melanophore scale in 1922 and an improved one in 1930. Without this the detailed analyses of colour change carried out by his school would not have been possible. After a few hours practice, a novice, with fig. 2 for reference, can read the melanophore indices (m.i.) of 12 animals without his average differing by more than 0·1 from that of an experienced supervisor.
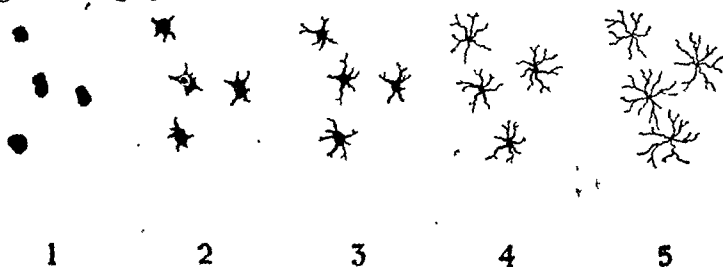
FIG. 2.—Melanophore Index (m.i.).

Three preparations permit easy reading of melanophores: (a) isolated skin immersed in saline, (b) perfused pithed specimens, and (c) intact or hypophysectomised animals with webs that can be quickly positioned on the microscope stage. Experience shows that only (c) yields results of quantitative value.

For assay of "B" by injection of extracts into intact or hypophysectomised animals,[1] *Phoxinus*,[2] *Rana*, and *Xenopus* have been used by various workers [Landgrebe *et al.*, 1941, 1943]. We find *Phoxinus* quite unreliable. Satisfactory assays can be made on *Rana*, but *Xenopus* is preferable for three reasons:

(a) *Ease of Reading.*—*Xenopus* has a much larger web than *Rana temporaria* and it can more rapidly be positioned on a microscope stage. Chromatophores other than melanophores are either absent or non-obtrusive. The apparent change of shape of the melanophore in transition from 1 to 5 on the melanophore scale is "smoother" and more uniform throughout the web.

(b) *Ease of maintaining Standardised Conditions.*—Temperature, humidity, and light have profound effects on chromatic function of amphibia. All three are easily standardised in an aquatic animal such as *Xenopus*. A serious objection to use of British *Rana* is the

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[1] The assay using *Anolis* (as with other reptiles) depends on macroscopic appearance because there are no webs.

[2] With this fish observation is made on erythrophore activity. It is still an open question whether erythrophores and melanophores are activated by the same pituitary autocoid [Landgrebe *et al.*, 1941, 1943].

Measurement of Melanophore Expansion or Skin Darkening.

This can be done by microscopic observation of the melanophores or by naked-eye estimation of the colour of the skin as melanophores

(a) m i = approx. 2.
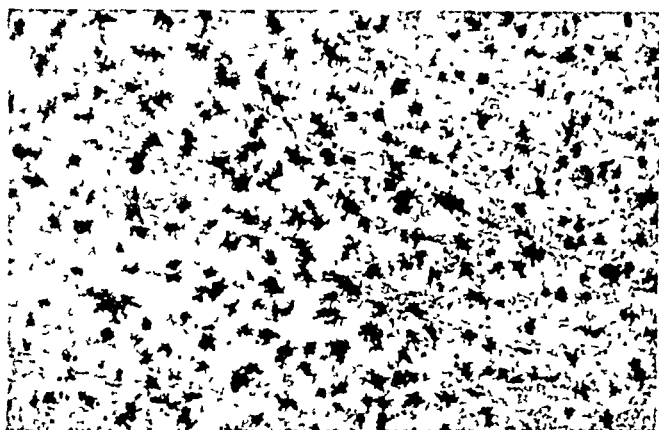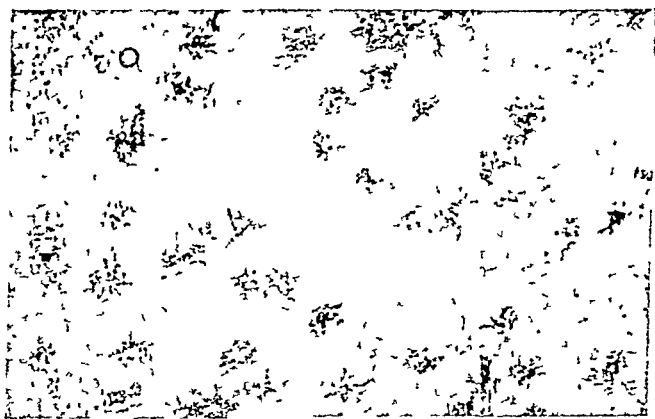
(b) m i = approx. 5

FIG. 1.—To show how a combination of reduced (i) melanophores per unit area and (ii) melanin per melanophore may result in a skin with melanophores equilibrated at m i = 2 being macroscopically darker than skin at m i = 5
Magnification of both photographs the same [1]

expand. The objections to the latter, and to Hill's photo-electric method of recording it, have been fully stated by Hogben [1936, 1942] and Neill [1940]; the overriding objection is the variation in melanophore density which may be so great (as, for example, in the animals

[1] We have *Xenopus* with less melanin than in (b), but these do not photograph well with the melanophores expanded.

evoke expansion (*i.e.* they are more sensitive). Animals that are exposed to conditions that cause prolonged expansion of the melanophores (intact animals on a black "background" or blinded animals in light, or animals with the *pars glandularis* removed) build up pigment, multiply their melanophores and decrease in sensitivity to "B" (fig. 3). In its incipient stages this loss and gain of sensitivity is not the same for all melanophores in one web. So that an animal with a varied "background" history spread over a week or so may react to an injection by some of the melanophores expanding to 2 and others to 5. It is clearly impossible to obtain an accurate assay with such animals. This is prevented by keeping the animals on a white "background" for a few weeks, after which the melanophores react similarly. Sensitivity of animals kept on a white "background" increases fairly rapidly for 12 months. Then the increase is less rapid and the responses are consistent over a period of weeks. The increase of sensitivity and decrease of pigment are both less rapid than the corresponding *decrease* in sensitivity and *increase* of pigment. Sensitivity and amount of pigment may be correlated so that the increased sensitivity that results from prolonged sojourn on a white "background" *may* be due to the reduced amount of pigment activated.

Respective Merits of Normal and Hypophysectomised Xenopus for Assay Purposes.—Assays of all fluids with unknown contaminants (*e.g.* from blood, urine, etc.) should first be done on intact animals which have a far higher resistance to histamine, foreign protein and other toxic substances. When dealing with extracts *not* derived from the pituitary, it is essential to obtain final figures from hypophysectomised animals. Substances occur in urine, etc., that evoke release of "B" from the test animal's own pituitary. There is no evidence for the presence of such substances in pituitary extracts prepared according to the standard procedure.

Simple extracts of the pituitary give similar results with normal and hypophysectomised *Xenopus*, and for such extracts intact animals are advocated. Reliable assays can then be obtained under certain known external conditions which four years' experience permits us to specify precisely (p. 9). Further advantages of using intact animals are: (*a*) they are easier to keep with unskilled assistance, and (*b*) *Xenopus* are in short supply and it is convenient to "borrow" animals for "B" assay from other uses such as pregnancy diagnosis. The quantitative data in this paper are from normal toads, but exactly similar results are obtained from hypophysectomised animals.

Respective Merits of Intraperitoneal and Dorsal Lymph Sac Injections.—We have used dorsal lymph sac (D.L.S.), intraperitoneal (I.P.), subcutaneous, and intravenous injections. Intravenous injections are extremely tedious and quite impracticable for routine work. *Xenopus* skin does not readily close over a skin puncture so subcutaneous injections are unreliable. Intraperitoneal injections may give erratic results: sometimes one or two of a group do not respond (probably due to extract entering the viscera). If, however, large numbers of test animals are used, fairly consistent results can be obtained. The

dominant rôle played by humidity: in about 50 per cent. of intact specimens, melanophores are always expanded in presence of water. When kept dry, survival is low.

. (c) *Length of Life.*—*Xenopus* lives indefinitely in the laboratory and its maintenance is easy. *Rana temporaria* can sometimes be kept alive for a few months but only with considerable attention. The advantages of the former are:

(i) Under conditions to be specified, their response to a given dose is consistent for long periods (fig. 3). Thus a rapid rough assay can be made and the appropriate dose chosen (p. 11).
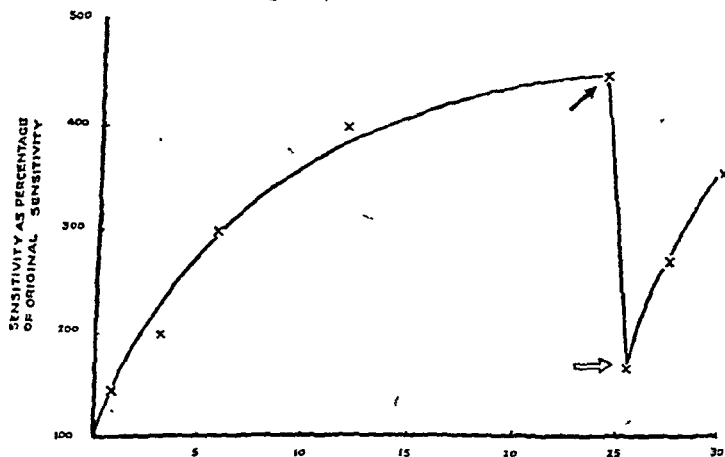
FIG. 3.—Graphic representation of the Effect of "Background" on the Sensitivity of *Xenopus* to Injection of "B"-containing Extracts.

All animals kept on a black "background" for several weeks before experiment: For the first 24 months of the experiment toads kept on a white "background." At → they were transferred to a black "background." At ⇒ they were transferred to a white "background." Points are the average sensitivity figures from same 12 toads. Sensitivity estimated at intervals shown by injecting each toad equilibrated on a white background with same dose of freshly made extract from the same sample of P.L.P. powder kept in a desiccator at 0° C.

(ii) Accurate reading of animals with many melanophores is difficult. This is one of the chief obstacles to accurate assay with the *Rana* available to us. *Xenopus* kept for long periods on a white background have fewer melanophores (fig. 1).

(iii) Amphibians exposed to conditions that evoke contraction of melanophores (intact animals on a white "background"[1] or completely hypophysectomised animals) gradually lose their pigment [*cf.* Dawes, 1941] and some melanophores disintegrate.[2] Animals so treated require less "B" to

[1] See Waring [1942], p. 124. By white "background" we mean a white container with overhead illumination. Similarly black "background" means black container with overhead illumination.

[2] Recent breeding experiments show that offspring from the same mating reared on the same background throughout development show great differences in degree of pigmentation.

of flattening is not directly proportional to the "B" content of the original powder [Landgrebe *et al.*, 1941, 1943].

(iii) Inert protein (*e.g.* presence of anterior lobe tissue) in the extracts flattens the time-response curve even when extraction is made by the standard method with dilute acid (fig. 5).

(iv) The second method takes longer than the first.

We, therefore, use the first criterion—peak m.i., attained—when comparing potency of extracts.
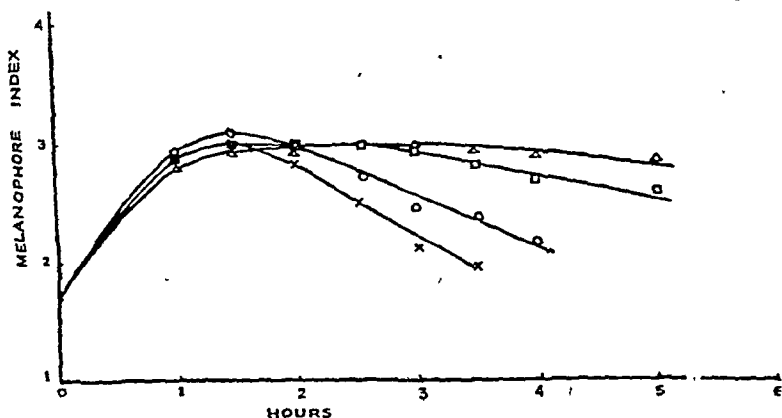
Fig. 5.—Responses of same Group of Intact *Xenopus* to Extracts of whole Gland and Posterior Lobe alone.

Doses adjusted to give approximately same peak m.i.   Each point is the average reading from 6 toads.   White background.   15° C.   All doses 0·25 c.c. D.L.S.

o = whole gland extracted by standard procedure with 0·25 per cent. acetic acid.

× = posterior lobe alone extracted by standard procedure with 0·25 per cent. acetic acid.

△ = whole gland extracted by standard procedure with 0·25 per cent. acetic acid, neutralised, adjusted to N/10 NaOH, heated in boiling-water bath for 10 minutes and then neutralised.

☐ = posterior lobe alone,   ditto.

III. Practical Details.

(a) *Care of Animals.*

Xenopus should be kept separately in white containers (*e.g.* 6 inches diameter, 8 inches high) half-filled with water and fitted with wire-mesh lids.   We keep them in a "dark room" thermostated at 16° C., with one 40-watt lamp above 24 containers.

At 16° C. *Xenopus* will sometimes eat even when being constantly handled as in assays.   Every six months or so we "rest" the animals at 20° to 25° C. when they feed voraciously and rapidly regain any loss of condition [Landgrebe, 1939].   Feeding once a week is sufficient and the containers must be cleaned out next day.

response curve after I.P. injection differs from that after D.L.S. injection and varies considerably for different groups of animals. Fig. 4 shows two things: (a) four to ten times the dose is required if I.P. injection is used, (b) for the peak responses 2·5 and 3·5, the ratio of the doses required by D.L.S. injection is 1 : 3·5, while for I.P. injection the ratio is only 1 : 2, i.e. I.P. injections give greater discrimination than D.L.S. injections. Unquestionably the most satisfactory route for injection is the *dorsal lymph sac*. It is absolutely reliable and the "relative dose" —"max. mel. index" curve (fig. 4)—is almost identical for different groups of test animals.
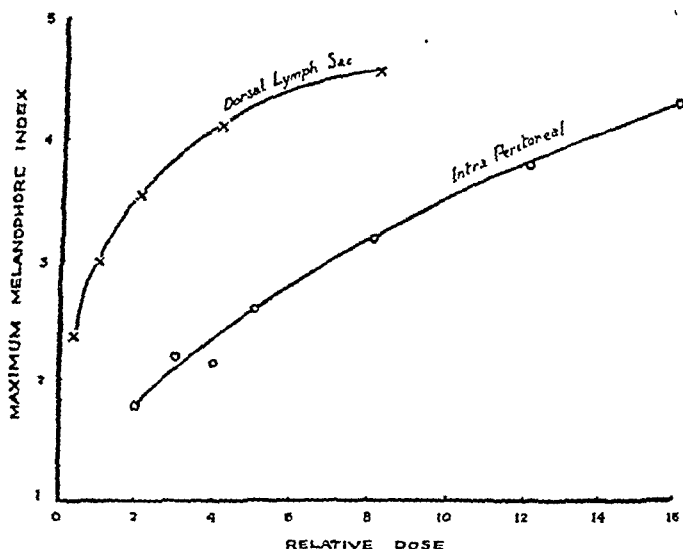
FIG. 4.—Dose Response Curve of the 18 *Xenopus* used to collect Data for Tables II and III.

×—×, Dorsal lymph sac.
o—o, Intra peritoneal.

Criterion for comparing Potency of Extracts.

There are two criteria for comparing potency of extracts: (a) peak m.i. attained, and (b) length of time between injection and re-assumption of complete pallor. Standard extracts of dried posterior lobe powders give similar results using either. We do not use the second criterion for the following reasons:—

(i) The time response curve is very flat towards the end and there is room for considerable error.

(ii) It is not applicable to extracts treated with caustic soda because this treatment flattens the time-response curve. The degree

IV. PERFORMANCE OF AN ASSAY.

(a) *Preliminaries*.

1. Select a large sample of posterior lobe powder preferably prepared from fresh gland and stored according to standard procedure [Burn, 1937]. Use this as substandard. There is no reason to suppose that such a preparation deteriorates even over a period of thirteen years [*cf.* 1st and 2nd International Standard powders, Dale, 1942, and Table II of this paper].

2. Prepare a standard extract of substandard powder with dilute acetic acid (B.P., 1932). Such extracts are stable for a few hours and then *sometimes* deteriorate rapidly. Stehle [1936] also noted this instability of *some* extracts. Solutions store well if boiled in ampoules, sealed and kept in the dark. At room temperature they are stable for at least three months and at 0° C. for at least six months. Alkali treated extracts must be standardised against extracts of international standard powder subjected to similar alkali treatment.

3. Divide 12–24 toads into two equal groups, A and B.

(b) *Standardisation of Substandard Powder*.

Inject suitable doses (refer to Table II) of the standard into test animals of group A and substandard into those of group B. On the
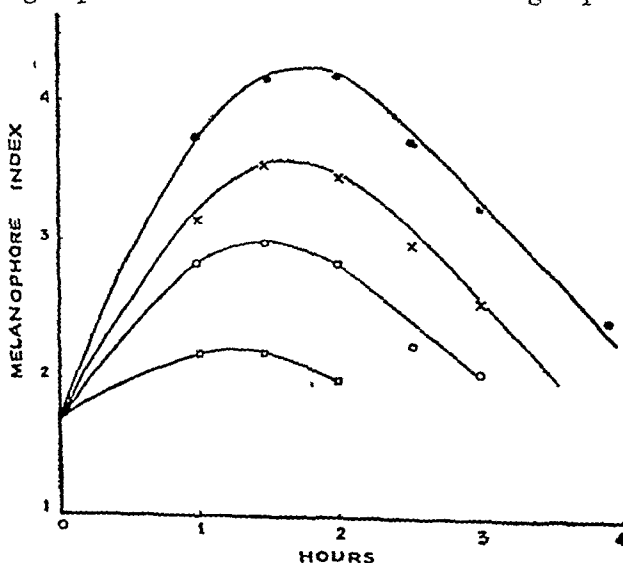
FIG. 6.—To illustrate the response of intact animals to graded doses of the same "B"-containing extract (supplied by Prof. Stehle). 15° C. White background. All injections ¼ c.c. D.L.S.

● =0·2 γ,   × =0·1 γ,   o =0·05 γ,   □ =0·025 γ Stehle's powder.

(b) *Miscellanea with Regard to Assay.*

(i) We have performed successful assays on animals weighing from 10 to 200 g., but most workers find toads weighing about 30 g. easiest to handle.

(ii) Dermal melanophores only are used. Animals kept on a white background for six months will have a convenient number of dermal and no epidermal melanophores. Otherwise, toads with many epidermals should be discarded as they lead to inaccuracies.

(iii) Aim at a dose that will give a peak m.i. around 3·0.

(iv) There is a slight gradient of melanophore expansion from the tip of the web proximally, so readings should be taken from the central portion of the large web to obtain a figure approximating to the average m.i. of the whole web.

(v) Reading is best done with 2/3 objective and ×10 eyepiece. Strength of microscope illumination must be adequate and constant.

(vi) Injections are made with a 2-inch gauge 6 needle, through the thigh muscles. This point of entry is used both for D.L.S. and I.P. injections.

(vii) As many as 20 D.L.S. injections can be made with water as a solvent. More than this often results in a large dark area appearing on the back ("jacketing") and the melanophore index in the web does not rise. This is because the injected activity does not leave the lymph sac owing to inactivation of the lymph heart. "Jacketing" can be avoided by using the following saline as a solvent:—

Total Content.				*Prepare as follows.*			
NaCl	.	. 8·3	g.	*Soln.* 1.	NaCl	.	. 8·5 g.
KCl	.	. 0·33	,,		KCl	.	. 0·34 ,,
CaCl$_2$	.	. 0·12	,,		H$_2$O	.	. 1000 c.c.
NaHCO$_3$	.	. 0·1	,,	*Soln.* 2.	CaCl$_2$6H$_2$O	.	. 0·24 g.
MgCl$_2$	.	. 0·1	,,		H$_2$O	.	. 10 c.c.
Dextrose	.	. 0·5	,,	*Soln.* 3.	MgCl$_2$6H$_2$O	.	. 0·22 g.
H$_2$O	.	. 1000	c.c.		H$_2$O	.	. 10 c.c.

Just before use mix: 100 c.c. of *Soln.* 1, 1 c.c. of *Soln.* 2, 1 c.c. of *Soln.* 3, and add 0·01 g. NaHCO$_3$ and 0·05 g. dextrose.

Animals that "jacket" in spite of the use of this saline have damaged lymph hearts and must be given I.P. injections.

(viii) Claims have been made that the volume of fluid used as a solvent for injected activity affects the response [Calloway *et al.*, 1942]. We cannot confirm this. Nevertheless it is advisable to standardise the volume injected. We find that 1 c.c. and 0·25 c.c. give identical responses and suggest the use of 0·25 c.c.

VII. Implications of the Observation that "B"/Pressor and "B"/Oxytocin Ratios are not the Same in all Posterior Lobe Powders as they are in International Standard Powder.

Data in Table I demand further examination on two issues:

1. *The Low "B"/Pressor Ratio of most Powders.*

There is convincing evidence that "B" emanates from the *pars intermedia* and pressor, oxytocic, and antidiuretic activities from the *pars nervosa*.

There is yet no evidence that ox pituitary powders *prepared according to standard procedure* have a pressor/oxytocic ratio different from that of I.S.P.P. Nor is there any indication that a relaxation of "standard" conditions (*e.g.* delay between killing and immersion of gland in acetone, etc.) results in differential loss of oxytocic and pressor activities.

Table I shows that the B/pressor ratio is not constant in various samples of powder. There may be two reasons for this:

(*a*) Since "B" and pressor come from different parts of the gland it may be that the size ratio of these two parts varies. So the discrepancy noted may be due to different quantities of "B" and pressor in the original gland. There is no evidence for this.

(*b*) That with these other powders some relaxation of the prescribed methods of preparation or storage may have resulted in greater loss of "B" than of pressor activity. There is no direct evidence for this either, but two reasons predispose us to consider it more likely than (*a*) above. They are: (1) close similarity of the "B" pressor ratio of the two international powders prepared in different countries at a thirteen years' interval, (2) in all powders, except apparently the Canadian S.P., where the "B" potency is low, the pressor figure is also below standard. Of the several ways by which relaxation of the

TABLE IV.—ACTIVITY EXPRESSED IN "INTERNATIONAL" MILLI-UNITS OF "B" PER MGM. OF POWDER.

	A.L.P.	P.L.P.	Whole gland.*
Commercial (Oxo) . . .	100	300	130
F. G. Y.	60	320	100
International preparation A.L.P.†	50	..	..
,, ,, P.L.P.†	..	2000	375

* Assuming the A.L.P. is five times the weight of its P.L.P.

† Although both these powders were produced by Armour, we have no information as to whether they were prepared from the same glands.

next day, inject the same dose of standard into B and of substandard into A. Repeat this procedure at three dose levels. This will yield data that can be used for (a) constructing a skeleton dose-response curve, (b) calculating the potency of substandard to about 10 per cent. (cf. Table II). To construct an accurate dose-response curve several dose levels must be used. Fig. 6 shows the kind of discrimination to expect between different doses.

If a large number of routine assays are not contemplated and a figure correct to about 20 per cent. is sufficient, a simpler procedure can be adopted. Inject group A with I.S.P.P. and group B with the unknown extract at one dose level (preferably a dose that raises the m.i. to about 3). Read off the result on fig. 4 of this paper. Experience shows that the sensitivity of toads varies considerably, but the dose-response graph of any group for *dorsal lymph sac* injections is not significantly different from that illustrated. This is strikingly demonstrated by comparison of graph 6 in Waring and Landgrebe [1941], which is based on observation of hypophysectomised toads by Waring in 1940, and which conforms to fig. 4 of the present paper, based on normal animals read by Landgrebe in 1942.

(c) *Assay of an Unknown Extract.*

It must again be emphasised that hypophysectomised animals must be used for other than pituitary extracts.

Prepare three or four trial doses of the unknown and inject each into one toad. At 16° C. read at $1\frac{1}{2}$ hours (peak rise). From the figures so obtained estimate a dose that will raise the average m.i. of a group to about 3·0. Avoid doses that raise the index above 4 because sometimes melanophores temporarily lose their ability to attain 5·0. Inject the unknown extract into group A and a comparable dose of substandard into group B. Reverse the groups next day. The response to substandard shows whether the toads are behaving true to form. If they are, the potency of the unknown extract can be read off on the dose-response curve (cf. Tables II and III).

V. Sample Assays.

(a) Table II is a reproduction of the final data from an assay to determine the number of L.W. units per mg. of international standard powder. The L.W. standard powder is that which has hitherto been used by us as a standard for "B" assays [Landgrebe and Waring, 1941]. Table II shows that 1 mg. of international standard powder contains 1000 L.W. units (i.e. 1 L.W. unit = 2 "international" units).

(b) Table III shows a comparison of first and second international standard powders. The second, which is now the standard, is 97 per cent. of the first.

TABLE III.—ASSAY TO DETERMINE THE RELATIVE "B" CONTENT OF THE 1ST AND 2ND INTERNATIONAL STANDARD POWDERS. All conditions same as in Table II.

Two groups (A and B) of 12 intact *Xenopus* from the same group as C and D of Table II.

Date.	First International Standard Powder.					Second International Standard Powder.					Second International Standard as percentage of First International Standard.
	Group of 12 toads.	Dose in milli-units.	Max. M.I.	Average max. M.I. from 24 toads.	"Relative dose" read from fig. 4.	Group of 12 toads.	Dose in milli-units.	Max. M.I.	Average max. M.I. from 24 toads.	"Relative dose" read from fig. 4.	
18.5.43	A	5·0	4·1	4·05	3·5	B	5·0	4·0	4·0	3·2	$\frac{3\cdot2}{3\cdot5} \times 100 = 91$
19.5.43	B	5·0	4·0			A	5·0	4·0			
20.5.43	A	2·5	3·2	3·2	1·25	B	2·5	3·2	3·15	1·2	$\frac{1\cdot2}{1\cdot25} \times 100 = 96$
21.5.43	B	2·5	3·2			A	2·5	3·1			
22.5.43	A	1·25	2·4	2·4	0·5	B	1·25	2·6	2·45	0·53	$\frac{0\cdot53}{0\cdot5} \times 100 = 106$
23.5.43	B	1·25	2·4			A	1·25	2·3			

standard procedure might lead to greater loss of "B" than of pressor activity, Table IV shows that we cannot explain the low "B" content in two examples by diffusion of activity into the anterior lobe.

2. *The Effect of various Activities on each other with Reference to Accuracy of Assays.*

The possibility that the *ratio* of pressor, oxytocic, and antidiuretic activities in substandards may be different is envisaged in Mem. 36 [1936]. This memorandum does not discuss the possibility that a disproportionate amount of one of the three activities might affect the assay of another. The relevant literature is meagre.

There is some evidence that oxytocin inhibits the coronary constriction caused by large doses of pressor, but no information is available to show that the pressor assay is significantly affected by a disproportionate ratio of pressor and oxytocin in the doses usually injected. Kamm *et al.* [1928] found that their pressor fraction evoked the same blood-pressure rise in the anæsthetised dog when injected alone or mixed with an equal number of oxytocin units. Smith [1943], using various mixtures of pressor and oxytocin, showed that the "pressor principle in preponderant amounts does not inhibit the action of the oxytocic on the isolated uterus."

There is no experimental evidence to show that the presence of "B" affects assays of pressor, oxytocic, or antidiuretic activities. Earlier work [Landgrebe and Waring, 1941] showed that the presence of pressor in injections of a "B" extract reduces the time taken for the melanophores to contract once the peak response is passed. To determine whether pressor reduces the *peak response* to "B" it is necessary to test the effect of pressor-free "B" before and after the addition of known amounts of pressor. Previously this was not possible because we had no pressor extract substantially free from "B." We now have an extract that contains less than 1 "I.U." of "B," 20 I.U. pressor and 100 I.U. of oxytocin. The amount of "B" in this extract was checked by treating it with caustic soda. Over 95 per cent. of the pressor and oxytocic activity was thus destroyed and the amount of "B" present after treatment was determined without the possible interfering effect of large doses of pressor and oxytocin. Experiments with this extract show that within the range of mixtures of neural lobe activities and "B" likely to be encountered, the former have no effect on the latter *when peak m.i. is the criterion of potency.*

We may conclude therefore that it is legitimate, if expedient, to keep one substandard of posterior pituitary in the laboratory for the assay of both neural lobe activities and "B," provided it is assigned a separate figure of potency relative to that in I.S.P.P. for each activity [*cf.* Mem. 36, 1936, p. 9].

VIII. SUMMARY.

1. Detailed instructions are given for assay of melanophore-expanding hormone using *Xenopus laevis* as a test animal.   10 per cent. accuracy is quickly and easily obtained.

2. The criterion of potency is the maximum melanophore index attained after injection of extracts into the dorsal lymph sacs of fully pale intact or hypophysectomised animals.

3. Intact *Xenopus* kept on a white "background" for long periods sustain an actual loss of pigment and their sensitivity to pituitary extracts is increased.

4. The first international standard posterior lobe powder contains 97 per cent. of the melanophore-expanding activity of the new standard.

5. We suggest that an international unit of melanophore activity be defined as that amount in 0·5 mgm. of the international standard powder.

6. The ratio of melanophore-expanding, pressor, and oxytocic activities is not the same in all posterior lobe powders.

7. The maximum melanophore index evoked by injection of a posterior lobe extract is not influenced by the amounts of pressor and oxytocin normally present.   So one substandard powder may be used for assay of all activities, provided a separate figure is assigned for the potency of each activity in terms of the corresponding activity of the international standard powder.

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FACTORS IN PANCREATIN WHICH INFLUENCE THE NUCLEO-PROTEIN CONTENT OF FIBROBLASTS GROWING *IN VITRO*. By J. N. DAVIDSON and C. WAYMOUTH. From the Physiology Department, University of Aberdeen.

IN the course of recent experiments on the chemical nature of the growth-promoting principles of embryo-extract, we reported [Davidson and Waymouth, 1943 *a, b*] that commercial pancreatin, which is prepared by extracting pancreas glands with dilute alcohol, appeared to contain factors which exert a profound influence on the growth of chick heart fibroblasts *in vitro*. These factors have now been partially purified. In this paper some of their properties and effects are described.

METHODS.

The roller-tube tissue-culture technique of Willmer [1942 *a*] was employed as described previously [Davidson and Waymouth, 1943 *a*]. Each roller-tube contained 6 fresh explants of the 9-day chick-embryo heart in 0·2 ml. fowl plasma. All tubes·were rotated in the incubator at 38° with 0·5 ml. Tyrode solution alone as fluid phase for 48 hours. The fluid was then removed and replaced by 0·5 ml. medium consisting of a suitable combination of Tyrode solution, embryo-extract and test substance, and the tubes replaced in the incubator. After a preliminary period of 1–3 hours some of the tubes were removed to give values for the initial amount of tissue present. The remainder were allowed to rotate for a further 24 or 48 hours or other suitable period. Control tubes with plasma but no tissue were also run. Determinations of nucleoprotein phosphorus (N.P.P.) were made at the beginning (time 0) and at the end (time *t*) of each test period as previously described, two or three roller-tubes being used at each time and with each test substance. The number of tubes which could be accommodated on the rotating drum in any test was limited to 20. Plasma blanks were therefore dispensed with in some experiments, after it had been established that with the preparations under test their use made no significant difference to the results. Growth was assessed by the increase in N.P.P. during the test period. This method was found to be valid for determining the growth-promoting activity of embryo-

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of a dense compact appearance with a sharply demarcated edge. At first sight they present the appearance often associated with degenerating cultures containing fat globules, but microscopic examination of the cells shows that no signs of degeneration are present.

Sections of the cultures were also made after fixation in formol-saline and embedding by the method described by Parker [1938]. The presence of the pancreatin factor alters the appearance of the cells considerably. As shown in Plate I (C and D) the control cultures (C) present the usual network appearance of elongated cells with oval nuclei, whereas the cultures grown in the presence of the pancreatin preparation (D) consist of well-nourished polyhedral cells with rounded nuclei. Mitotic figures are clearly seen.

2. *Nucleoprotein Content of the Cultures.*—As in earlier work [Davidson and Waymouth, 1943 a], direct comparisons of the effects of two substances on the N.P.P. of the cultures are normally made where control and test cultures are grown in the same plasma at the same time. In Table I the complete results of three such tests are quoted, while in Tables II and III the results are summarised in terms of the alteration in N.P.P. during the test period.

TABLE I.—EXAMPLES OF TISSUE CULTURE TESTS WITH N.P.P. VALUES FOR TUBES WITH AND WITHOUT TISSUE AT THE BEGINNING (3 HOURS) AND END (48 HOURS) OF THE TEST PERIOD.

Test No.	Test substance in fluid phase in roller-tube.	N concentration mg./100 ml.	N.P.P. μg. per roller-tube.				Change.	Morphological changes.
			Initial.		Final.			
			Plasma + tissue.	Plasma alone.	Plasma + tissue.	Plasma alone.		
61	Tyrode solution alone .	0	1·05 1·20	0·26	1·03 1·03	0·24	−0·07	−
	Embryo-extract + Tyrode	30 + 0	1·33 1·29	0·41	1·44 1·73	0·29	+0·39	−
44	Embryo-extract + Tyrode	30 + 0	1·63	0·29	2·13 1·90	0·30	+0·37	−
	Embryo-extract + crude pancreatin preparation	30 + 16	1·66	0·32	2·80 2·27	0·28	+0·91	+ +
82	Embryo-extract + Tyrode	30 + 0	1·48	0·36	1·79 1·73	0·32	+0·32	−
	Embryo-extract + phenol soluble fraction (i)	30 + 2	1·25 1·65	0·34	2·04 1·92 1·77	0·33	+0·47	−
	Embryo-extract + phenol soluble fraction (ii)	30 + 20	1·54 1·38	0·37	2·20 1·90 1·51	0·30	+0·48	+ +

extract, and its use was therefore extended to experiments with other growth-promoting substances. For example, in a series of 10 tests with embryo-extracts containing 30 mg. N/100 ml., N.P.P. was estimated at 3 and 48 hours (time 0 and time t) in a total of 35 roller-tubes with tissue, *i.e.* in 215 pieces of tissue, and in the corresponding plasma blanks. A mean rise of 0·39 μg. per roller-tube (standard deviation = ±0·045, standard error = ±0·016) was found with this concentration of extract, in spite of the wide range of (corrected) initial values (0·86–1·53 μg. N.P.P.) in the series.

The pancreatin preparations were thermostable. Aqueous solutions were therefore sterilised by heating for 10 minutes at 100° and treated with an equal volume of sterile double-strength Tyrode solution before being added to the cultures.

The factors can be prepared from fresh ox pancreas, but a more convenient starting material is commercial pancreatin. Crude solutions were prepared at first by a method similar to that used by Dubos and Thompson [1938] for the preparation of crude ribonuclease, by extracting commercial pancreatin with 50 per cent. acetone and increasing the concentration of acetone in the extract to 67 per cent. The oily precipitate was dissolved in water, extracted with ether, and heated to 100° for 10 minutes. The precipitate which appeared was centrifuged down and discarded. The supernatant fluid was dialysed in cellophane sacs, sterilised by heat and treated with an equal volume of double-strength Tyrode solution.

Later it was found more convenient to incubate commercial pancreatin overnight with water, filter the mixture, heat the filtrate to 100° for 10 minutes, cool, discard the precipitate and treat the supernatant fluid with 6 vols. ethanol. The sticky precipitate which fell out was washed with ethanol, then ether, and dried. Portions were dissolved in glass-distilled water as required, sterilised by heat, and treated with an equal volume of double-strength Tyrode solution.

RESULTS.

1. *Appearance of Cultures.*—The appearance of cultures grown in embryo-extract in the presence and absence of the material from pancreatin is shown in Plate I (A and B). As the curvature of the roller-tubes renders accurate photography difficult, the tube, filled with formol-saline fixative, was immersed in a trough of water with a glass bottom and photographed through a low-power microscope. The control cultures grown in embryo-extract alone present the normal appearance of proliferating fibroblasts, and a fine halo of new growth surrounds the remnant of the original explant. Cultures grown in embryo-extract to which the test material had been added show abundant growth of quite a different type. They are rather smaller in area, and

of a dense compact appearance with a sharply demarcated edge.   At first sight they present the appearance often associated with degenerating cultures containing fat globules, but microscopic examination of the cells shows that no signs of degeneration are present.

Sections of the cultures were also made after fixation in formol-saline and embedding by the method described by Parker [1938].   The presence of the pancreatin factor alters the appearance of the cells considerably.   As shown in Plate I (C and D) the control cultures (C) present the usual network appearance of elongated cells with oval nuclei, whereas the cultures grown in the presence of the pancreatin preparation (D) consist of well-nourished polyhedral cells with rounded nuclei.   Mitotic figures are clearly seen.

2.   *Nucleoprotein Content of the Cultures.*—As in earlier work [Davidson and Waymouth, 1943 a], direct comparisons of the effects of two substances on the N.P.P. of the cultures are normally made where control and test cultures are grown in the same plasma at the same time. In Table I the complete results of three such tests are quoted, while in Tables II and III the results are summarised in terms of the alteration in N.P.P. during the test period.

TABLE I.—EXAMPLES OF TISSUE CULTURE TESTS WITH N.P.P. VALUES FOR TUBES WITH AND WITHOUT TISSUE AT THE BEGINNING (3 HOURS) AND END (48 HOURS) OF THE TEST PERIOD.

Test No.	Test substance in fluid phase in roller-tube.	N concentration mg./100 ml.	N.P.P. μg. per roller-tube.				Change.	Morphological changes.
			Initial.		Final.			
			Plasma + tissue.	Plasma alone.	Plasma + tissue.	Plasma alone.		
61	Tyrode solution alone .	0	1·05 1·20	0·26	1·03 1·03	0·24	−0·07	—
	Embryo-extract + Tyrode	30 + 0	1·33 1·29	0·41	1·44 1·73	0·29	+0·39	—
44	Embryo-extract + Tyrode	30 + 0	1·63	0·29	2·13 1·90	0·30	+0·37	—
	Embryo-extract + crude pancreatin preparation	30 + 16	1·66	0·32	2·80 2·27	0·28	+0·91	+ +
82	Embryo-extract + Tyrode	30 + 0	1·48	0·36	1·79 1·73	0·32	+0·32	—
	Embryo-extract + phenol soluble fraction (i)	30 + 2	1·25 1·65	0·34	2·04 1·92 1·77	0·33	+0·47	+
	Embryo-extract + phenol soluble fraction (ii)	30 + 20	1·54 1·38	0·37	2·20 1·90 1·51	0·30	+0·48	+ +

As previously reported, cultures grown in Tyrode solution alone as fluid phase during the test period usually show a slight fall in N.P.P. (*e.g.* Table I, Test 61). On the other hand, as shown above with embryo-extract (containing 30 mg. per cent. total N), an appreciable rise in N.P.P. (0·35–0·45 μg. per roller-tube) occurs (Tables I and II). Much greater increases are found when this concentration of embryo-extract is supplemented by the pancreatin material (Table I, Test 44; Table II). Crude preparations are rich in ribonuclease, but the presence of this enzyme alone is not responsible for the effect. It has already been shown that crystalline ribonuclease produces little or no effect on the growth-promoting properties of embryo juice [Davidson and Waymouth, 1943 *a*].

TABLE II.—EFFECT OF CRUDE PANCREATIN PREPARATIONS ON THE N.P.P. OF TISSUE CULTURES. EACH FIGURE IS DERIVED FROM SEVERAL ROLLER-TUBES. PLASMA BLANKS HAVE BEEN SUBTRACTED.

Test No.	Test substance.	Nitrogen concentration mg./100 ml.	Test period, hours.	Change in N.P.P. μg. per roller-tube.	Morphological changes.
44	Embryo-extract + Tyrode	30 + 0	48	+ 0·37	–
	Embryo-extract + crude pancreatin preparation	30 + 16	48	+ 0·91	+ +
45	Embryo-extract + Tyrode	28 + 0	48	+ 0·41	–
	Embryo-extract + crude pancreatin preparation	28 + 8	48	+ 0·54	+ +
	Tyrode + crude pancreatin preparation	0 + 8	48	+ 0·05	–
49	Embryo-extract + Tyrode	32 + 0	48	+ 0·45	–
	Embryo-extract + crude pancreatin preparation	32 + 15	48	+ 1·28	+ +
51	Embryo-extract + Tyrode	20 + 0	24	+ 0·36	–
	Embryo-extract + crude pancreatin preparation	20 + 15	24	+ 0·82	– –

Although the factor causing the morphological changes is stable at 100° in neutral or slightly acid solution, its power to produce the characteristic dense appearance in the cultures is destroyed by heating at 100° for 30 minutes at pH 8–10. The ribonuclease activity of the material is completely lost during this treatment.

3. *Purification of the Material.*—It has been found that the material can be partially purified by extraction from aqueous solution with phenol, solution of the phenol extract in ether and extraction of the ethereal phenol solution with water. The activity passes back into the

aqueous layer.   During phenol extraction most of the colour and about 40 per cent. of the nitrogen and 20 per cent. of the P present in the crude extract pass into the phenol layer.   The residue does not produce the characteristic morphological changes, although it may stimulate culture growth to some extent and cause a slight rise in N.P.P. above that produced by embryo-extract alone.   The purification process is as follows:—

Twenty-five grams of commercial pancreatin are mixed with 120 ml. of distilled water and are left overnight in the incubator at 37°.   The mixture is then heated at 100° for 10 minutes, cooled, and centrifuged. The slightly turbid supernatant fluid is treated with 6 volumes of ethanol, and the precipitate allowed to settle overnight in the refrigerator. It is then centrifuged down, and washed with ethanol and ether.   Weight 500–700 mg.   This material is deliquescent and is stored in a desiccator. For the next stage 100 mg. are dissolved in 10 ml. water, brought to 100°, cooled and centrifuged.   The precipitate is discarded and the supernatant fluid is extracted with three successive 3-gram portions of phenol.   At each extraction the phenol layer is separated by centrifuging.   The phenol extracts are combined and centrifuged at 3000 r.p.m. for 15 minutes.   A layer of white material which settles out on top of the phenol is discarded.   The clear phenol extract is dissolved in 100 ml. ether and the ethereal phenol solution is then extracted four times with small portions of water.   The clear yellow aqueous extracts are combined (total volume about 15 ml.), and extracted three times with small amounts of ether to remove the last traces of phenol.   The aqueous solution is then heated on the water-bath to remove traces of ether and to bring the volume down to about 5 ml.   The aqueous solution is cooled and centrifuged to remove a small amount of insoluble matter. · Six volumes of ethanol are then added ˅and the mixture allowed to stand in the refrigerator for an hour.   The precipitate is then centrifuged down, washed with ethanol and ether and dried in vacuo.   The yield is about 10–20 mg.

4. *The Effect of the Phenol Soluble Fraction (P.S.F.) on the Growth of the Cultures.*—The addition of the P.S.F. to embryo-extract in the tissue culture medium caused, as with the crude material, a greater increase in N.P.P. than the same concentration of embryo-extract alone (Table I, Test 82; Table II, Tests 77, 102, 103, 104).   Low concentrations of the material, although able to cause the changes in the morphology of the cultures, did not appreciably increase the power of a high concentration of embryo-extract (N = 30) [1] to raise the N.P.P.   An experiment with dilute embryo-extract (N = 3·6) and varying concentrations of the P.S.F. suggested that the effects of the extract and of the factor on the N.P.P. are really independent and additive.   Embryo-extract alone at this dilution (N = 3·6), although it gives rise to marked migrational

[1] N concentration in mg./100 ml. of the fluid phase added to the cultures.

outgrowth, does not significantly increase the N.P.P. of the cultures. but with the addition of a high concentration of the P.S.F. (N=100), the rise of N.P.P. in the 48-hour growth period was 0·65 μg., or more than 50 per cent. of the initial N.P.P. (1·32 μg.). The effect of increasing concentrations of the P.S.F. in the presence of embryo-extract of N=3·6 is shown in fig. 1 (Test 103).

The results of tests with a series of concentrations of the P.S.F., without embryo-extract, confirmed the capacity of this fraction alone to cause a rise in N.P.P., and showed that the magnitude of the effect is dependent on the amount of fraction present. With one sample at N=150, an increase in N.P.P. equal to about 100 per cent. of the initial
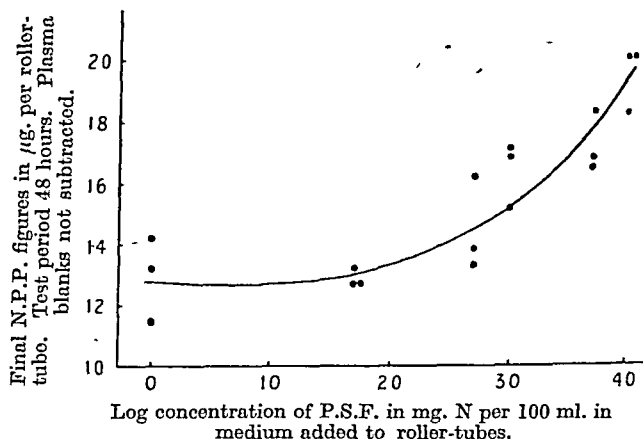
Fig. 1.—Effect of varying concentrations of P.S.F. with dilute embryo-extract (N=3·6) on the N.P.P. of cultures in a single experiment.

value was obtained in 48 hours. Lower concentrations produced proportionately smaller increases (figs. 2 and 3).

The characteristic alterations in the morphology of the cultures, observed ·with the P.S.F. and embryo-extract, were also found with the P.S.F. alone (Plate I, E and F). It appears that neither these morphological changes nor the effect on the N.P.P. require the presence of embryo-extract. Cells damaged during the preparation of the heart explants will release into the medium disintegration products which constitute in effect an embryo-extract, but these are not likely to be present, after the preliminary two-day period of washing in Tyrode solution alone, in sufficient amount to play any part in these effects. If traces of such cell products are in fact essential to the activities observed, their presence could not be entirely eliminated under the experimental conditions, for proliferating tissue cultures are bound to contain some disintegrating cells, continually releasing such products in small amounts.

It was observed that at the end of the 3 hours usually allowed for ·

the attainment of equilibrium ("time 0"), the N.P.P. with high concentrations of the P.S.F. was greater than that with Tyrode solution alone. Small differences of this kind are found with embryo-extract
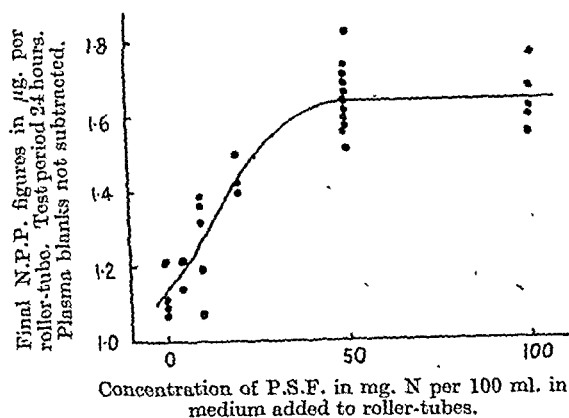
FIG. 2.—Effect of varying concentrations of P.S.F. (without embryo-extract) on the N.P.P. of cultures in several experiments.
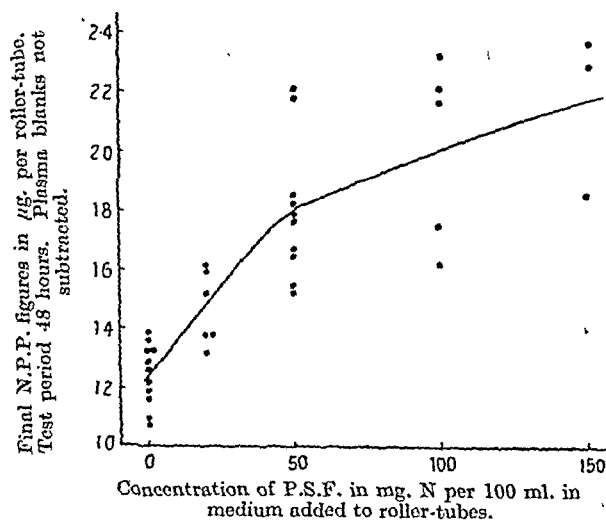
FIG. 3.—Effect of varying concentrations of P.S.F. (without embryo-extract) on the N.P.P. of cultures in several experiments.

and other nucleoprotein-containing media, owing to adsorption on the plasma coagulum, and correction is made for them by the determination of N.P.P. in the "plasma blanks." Since a control experiment showed that no corresponding rise occurred in tubes containing plasma and the P.S.F. but no tissue, the increase in N.P.P. detected in 3 hours, and

clearly demonstrated in 6 hours, must therefore be due to an actual increase in nucleic acid in the tissue. This increase very probably precedes cell division and is in agreement with the observation of Willmer [1942 b] that with strong concentrations of embryo-juice "there is a considerable rise (in N.P.P.) during the first 3 hours, so that the nucleoprotein increase anticipates the onset of nuclear division by some hours and more closely parallels in time the general increase in cellular activity." The altered appearance of the cultures is also produced at an early stage, and can be detected at 6 hours.
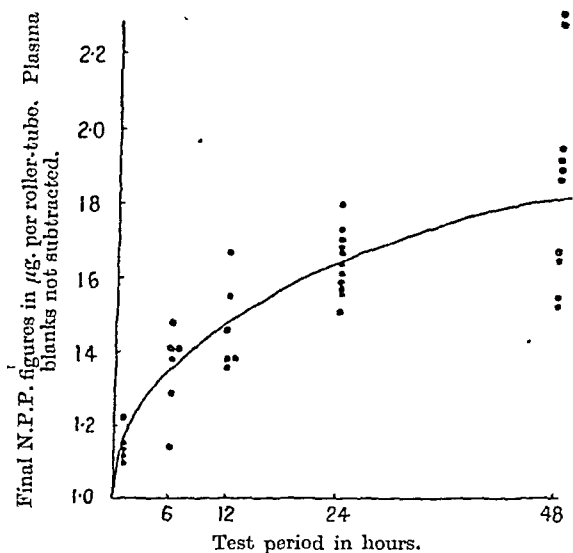
FIG. 4.—Effect of P.S.F. (without embryo-extract) on the N.P.P. of cultures at varying times in several experiments. The medium added to the roller-tubes contained the P.S.F. at a concentration of 50 mg. N per 100 ml.

The results of N.P.P. determinations after the application of the P.S.F. at a concentration of N = 50 for varying lengths of time are shown in fig. 4. The points plotted are derived from several experiments and relate to three different batches of material. Although there is a wide variation in the individual values, it is clear that a progressive rise in N.P.P. occurs. The time intervals selected are too great to detect whether the rise is in fact a continuous one or whether there is a step-wise increase, associated with the cycles of mitosis.

The variation of N.P.P. with concentration of P.S.F. is shown in figs. 2 and 3. Especially with high concentrations the rise in N.P.P. in 48 hours is considerably greater than that produced by the same concentration in 24 hours. The initial N.P.P. may even be doubled. Such an increase is as great as can be obtained in the same time with a very potent preparation of embryo-juice, but still greater increases can

be obtained when both embryo-juice and P.S.F. are present (*e.g.* Test 123, Table III).

Although the P.S.F. can produce a sharp rise in N.P.P. in a short time, it does not appear to be able to maintain the growth of cultures over long periods in the same way as does embryo-juice. We have not attempted to maintain a strain of fibroblasts through several passages with the aid of the material. Roller-tube cultures in which the P.S.F. was renewed daily for 5 days showed a lower N.P.P. content than did cultures grown for only 48 hours without renewal (Table III, Test 116).

The power of the purified material, as well as that of the crude preparations, to produce the changes in the morphology of the cultures is destroyed by heating at 100° for 30 minutes at pH 8–10. The same result is obtained with material autoclaved for 20 minutes at 132°. Such material is still able in the absence of embryo-extract to produce a very appreciable rise in N.P.P., only slightly less than that produced by the untreated purified factor (Table III). The addition of crystalline ribonuclease does not restore the ability of the treated material to produce the dense type of culture (Table III, Test 119).

5. *The Chemical Nature of the Phenol Soluble Fraction.*—The partially purified material is a light brown non-deliquescent powder freely soluble in water, acids, and alkalies. The aqueous solution is slightly acid in reaction and shows a blue fluorescence by ultra-violet light. It gives positive biuret and ninhydrin tests. Trichloracetic acid produces a slight turbidity, and tungstic and phosphotungstic acids heavy precipitates. The material gives faint precipitates with lead and mercury salts, and heavy precipitates with picric and flavianic acids. Tests for sulphur, pentoses, and desoxypentoses (diphenylamine test) are positive. A trace of ribonuclease activity is still present. The material responsible for the dense type of growth dialyses very slowly through cellophane membranes and is partially precipitated by ammonium sulphate at 0·8 saturation. It is adsorbed readily by alumina, charcoal, kaolin, and Fuller's earth, but poorly by Decalso.

Several samples of commercial pancreatin from different sources have been used as starting material. Even with the same sample the purification process did not give a product of constant composition. Different batches of purified material contain 8·3–13·6 per cent. N and 1·6–2·7 per cent. P. In one sample, 13 per cent. of the total N was in the form of amino N as determined by the gasometric method of Van Slyke [1929]. After hydrolysis in 5 N HCl at 100° for 24 hours the amino N rose to 60 per cent. of the total N, and hydrolysis for a further 24 hours caused no further increase. About 20 per cent. of the P is liberated at pH 8·4 in inorganic form by the action of snake venom containing an active 5-nucleotidase [Gulland and Jackson, 1938]. Estimation of total purine N by the method of Graff and Macula [1935] showed that

TABLE III.—EFFECT OF VARIOUS PREPARATIONS ON THE N.P.P. OF TISSUE CULTURES. EACH FIGURE IS DERIVED FROM SEVERAL ROLLER-TUBES. PLASMA BLANKS NOT SUBTRACTED.

Test No.	Test substance.	Nitrogen concentration, mg./100 ml.	Test period, hours.	Change in N.P.P. μg. per roller tube.	Morphological changes.
77	Embryo-extract (E.E.) + Tyrode	20 + 0	48	+0·25	–
	E.E. + phenol soluble fraction (P.S.F.)	20 + 10	48	+0·40	+ +
102	E.E. + P.S.F.	30 + 44	48	+0·77	+ +
	E.E. + P.S.F.	30 + 10	48	+0·60	+ +
	E.E. + P.S.F.	30 + 2	48	+0·51	+ +
	E.E. + P.S.F.	30 + 0·5	48	+0·48	+
	E.E. + P.S.F.	30 + 0·1	48	+0·42	(+)
	E.E. + Tyrode.	30 + 0	48	+0·42	–
103	E.E. + P.S.F.	3·6 + 100	48	+0·65	+ +
	E.E. + P.S.F.	3·6 + 50	48	+0·42	+ +
	E.E. + P.S.F.	3·6 + 10	48	+0·33	+
	E.E. + P.S.F.	3·6 + 5	48	+0·13	+
	E.E. + P.S.F.	3·6 + 0·5	48	−0·02	(+)
	E.E. + Tyrode.	3·6 + 0	48	−0·05	–
104	Tyrode + P.S.F.	0 + 50	48	+0·54	–
	E.E. + P.S.F.	2·5 + 50	48	+0·56	+
	E.E. + P.S.F.	10 + 50	48	+0·76	+
	E.E. + P.S.F.	25 + 50	48	+0·98	+ +
123	E.E. + P.S.F.	50 + 50	48	+1·09	+ +
115	P.S.F.	50	48	+0·87	+
	P.S.F. (autoclaved).	50	48	+0·90	–
116	P.S.F.	100	48	+1·02	+ +
	P.S.F.	100	120	+0·59	
	P.S.F. (autoclaved).	100	48	+0·85	+
117	P.S.F.	25	48	+0·19	+ +
	P.S.F. (autoclaved).	25	48	+0·08	–
119	P.S.F.	67	48	+0·76	+ +
	P.S.F. (autoclaved).	67	48	+0·61	–
	P.S.F. (autoclaved) + crystalline ribonuclease	67	48	+0·56	–
118	"Padutin"	50	48	+0·95	–
	"Padutin"	100	48	+0·32	–
120	"Padutin"	25	48	+0·60	–
	"Padutin"	75	48	+0·56	–
121	"Examen"	40	48	+0·11	–
	"Examen"	14	48	+0·42	–
	Peptone.	50	48	+1·27	–
	Peptone.	20	48	+0·86	–
	Yeast extract.	50	48	+0·56	–
122	Boiled kidney extract	25	48	+0·56	–
125	Boiled kidney extract	25	48	+0·97	–

10·4 per cent. of the total N was present as purine N. Analysis by the method of Kerr [1940] revealed that almost the whole of the purine N was present in the form of nucleotide, nucleoside, and free purine, mostly as nucleotide.

The phosphorus was resistant to alkaline hydrolysis. No inorganic P was split off after incubation with 0·25 N NaOH at 37° in the course of 96 hours.

6. *The Effect of other Substances on the Cultures.*—A large number of known substances has been tested to see whether they were capable of producing the morphological changes in the cultures. They included crystalline insulin, Witte's peptone, sulphur-containing compounds such as glutathione, thiourea, and thiohydantoin, histamine, ribonucleic acid prepared from pancreas [Jorpes, 1934], from liver [Davidson and Waymouth, unpublished], and from yeast, all four mononucleotides of yeast nucleic acid [prepared according to Steudel and Peiser, 1922, and Jones and Perkins, 1924], pyrimidine desoxyribosides [Levene and Bass, 1931], adenosine, guanosine, yeast adenylic acid, muscle adenylic acid, the amino acid mixture described by Fischer [1941 a], and phosphoprotein prepared from casein [Levene and. Hill, 1933]. None of them produced an effect similar to that associated with the pancreatin factor.

Some of the properties of the P.S.F. bear a resemblance to those of the anti-anæmic principle of liver as described by Karrer [1941], Erdos [1942], and Subbarow [1942], but a specimen of liver extract (Examen) produced none of the characteristic cellular changes although it brought about a small rise in N.P.P. (Table III, Test 121). Boiled ox-kidney extract prepared by the method of Fischer and Astrup [1942] caused both cell migration and a rise in N.P.P. without, however, producing the characteristic dense appearance (Table III, Tests 122, 125). A similar effect was produced by a preparation of the material obtained by the irradiation of living yeast cells [Davidson, 1940], and by commercial peptone (Table III, Test 121).

The vasodilator substance kallikrein [Dale, 1933] differs from our material in being thermolabile, but as it is also derived mainly from the pancreas [Frey, Kraut, and Schultz, 1930; Frey, 1932] it was thought advisable to examine its effects on the tissue cultures. A commercial preparation of kallikrein from pancreas (Padutin, Bayer) sterilised by heating for 10 minutes at 100° was tested out on the cultures at varying nitrogen concentrations and was found to bring about a very marked rise in N.P.P. (Table III, Tests 118, 120). It did not, however, appear to contain the thermostable material responsible for the morphological changes.

Crude preparations of ribonuclease made from pancreatin would also contain the enzyme lecithinase A which hydrolyses lecithin to lysolecithin [Delezenne and Fourneau, 1914], and which, like ribo-

nuclease, is remarkably stable to heat in slightly acid solution [Hughes, 1935; Gronchi, 1936]. A preparation of lecithinase A made from pancreatin by the method of Gronchi [1936] was able to bring about the morphological changes in the cultures. As such a preparation is far from pure, the effect of lecithinase from another source, snake venom, was investigated. Hughes [1935] has shown that lecithinase from snake venom will survive prolonged boiling at pH 5·9, although in slightly alkaline solution it is easily destroyed by heat. A sample of Russell's viper venom was dissolved in water and sterilised by heating at 100° for 10 minutes. A large amount of denatured protein which appeared during the heating was centrifuged down and discarded. The supernatant fluid was tested at several dilutions (7·5, 0·75, and 0·075 mg. N/100 ml.) in the presence of embryo-extract and found to produce the characteristic morphological picture even at the highest dilution.

DISCUSSION.

In considering the effects produced by the pancreatin factor or factors it is important to remember Williams's [1941] warning to distinguish "between an extract which contains some biologically active principle and a preparation which contains a single biologically active substance." Our material obviously falls into the former category.

Kazal, Westfall, Ciereszko, Risley, and Arnow [1942] have recently described a material prepared from pancreas which White and Sayers [1942] have shown to exert a powerful growth-promoting effect when fed to young rats. This material, prepared by extracting frozen beef pancreas with acid-alcohol and then acetone and pulverising the residue, is of course very crude in comparison with our preparation but would probably nevertheless contain the same factors. Daily intraperitoneal injection of 8 mg. of our material into weanling rats over a period of several weeks caused no difference in growth rate as compared with controls.

The solubility of the active material in phenol gives but little clue to its composition since some proteins and their derivatives and also nucleotides are soluble in this reagent. Phenol has been used as a solvent in the purification of antigens [Palmer and Gerlough, 1940; Morgan and Partridge, 1941], of anterior pituitary hormones [Freud, Laqueur, and Mühlbock, 1939], of the anti-anæmic principle of liver [Karrer, 1941], and of nucleotides [Warburg and Christian, 1938; Emmerie, 1938].

The properties of the purified material suggest the presence of protein and nucleic acid disintegration products which may be responsible, as nutrients, for the nucleoprotein synthesis. Similar large increases in N.P.P. have been found with protein breakdown products such as

peptones (Table III) and the phosphopeptone of casein. The growth-
promoting action of protein disintegration products has been known
for some time [Carrel and Baker, 1926; Willmer and Kendal, 1932
and has been emphasised by the recent work of Fischer [1941 a and b
1942]. The "X-substance" which Willmer and Kendal [1932] found
to stimulate tissue cultures is also apparently a protein disintegration
product, but differs from our substance in being insoluble in pure water
Although these heat-stable substances do produce stimulation of
growth, their action is to amplify the effect of embryo-extract rather
than to produce the dense type of culture with exceptionally high
nucleoprotein content characteristic of the action of the pancreati
material. The growth-promoting action of proteoses, peptones, an
polypeptides in combination with nucleotides is envisaged by Needham
[1942], who suggests "that a substance of nucleotide structure might
act either as a carrier for the requisite peptone 'bundle' . . . or els
possibly as a 'bricklaying' mechanism at the site of protein synthesis.
The action in the present instance is, however, not merely a quantitativ
increase in nucleoprotein synthesis during the normal process of growth
The qualitative change in the morphological character of the cel
must also be taken into account. That the material exerts its effe
by the action of at least two components is suggested by some of th
experimental evidence. After autoclaving, for instance, the materi
still retains most of its power to cause nucleoprotein synthesis, althoug
it can no longer produce the dense type of culture. Again the den
appearance of the cultures is very marked in concentrations of fact
which are too low to produce any detectable change in N.P.P. Th
suggests an enzymatic effect. Trypsin, which may stimulate t
growth of adult tissue *in vitro* [Simms and Stillman, 1937], would appe
to be connected only indirectly, if at all, with the factor since it wou
scarcely survive the purification process. Commercial pancreatin is
course rich in trypsin, which may no doubt play a part in producing t
protein disintegration products which compose the crude material, b
the active agent (or agents) is obviously resistant to tryptic digestic
as, for example, are the phosphopeptones [Lowndes, Macara, a
Plimmer, 1941; Rimington, 1941; Posternak and Pollaczec, 1941].

It is possible that one effect of the P.S.F. is not directly on the ce
of the culture but on the plasma clot. Such an action would presumab
be enzymatic in nature, although the purification process is sufficien
drastic to destroy all but the most resistant of enzymes. Ribonucle
activity is still evident in the P.S.F., but as far as can be ascertaine
ribonuclease, which is a remarkably heat-resistant enzyme, appears
have little connection with the activity. It is, however, destroyed
heating in alkaline solution.

The enzyme lecithinase A possesses a resistance to heat similar
that of ribonuclease. The activity of preparations of lecithinase fr

nuclease, is remarkably stable to heat in slightly acid solution [Hughes, 1935; Gronchi, 1936]. A preparation of lecithinase A made from pancreatin by the method of Gronchi [1936] was able to bring about the morphological changes in the cultures. As such a preparation is far from pure, the effect of lecithinase from another source, snake venom, was investigated. Hughes [1935] has shown that lecithinase from snake venom will survive prolonged boiling at pH 5·9, although in slightly alkaline solution it is easily destroyed by heat. A sample of Russell's viper venom was dissolved in water and sterilised by heating at 100° for 10 minutes. A large amount of denatured protein which appeared during the heating was centrifuged down and discarded. The supernatant fluid was tested at several dilutions (7·5, 0·75, and 0·075 mg. N/100 ml.) in the presence of embryo-extract and found to produce the characteristic morphological picture even at the highest dilution.

DISCUSSION.

In considering the effects produced by the pancreatin factor or factors it is important to remember Williams's [1941] warning to distinguish "between an extract which contains some biologically active principle and a preparation which contains a single biologically active substance." Our material obviously falls into the former category.

Kazal, Westfall, Ciereszko, Risley, and Arnow [1942] have recently described a material prepared from pancreas which White and Sayers [1942] have shown to exert a powerful growth-promoting effect when fed to young rats. This material, prepared by extracting frozen beef pancreas with acid-alcohol and then acetone and pulverising the residue, is of course very crude in comparison with our preparation but would probably nevertheless contain the same factors. Daily intraperitoneal injection of 8 mg. of our material into weanling rats over a period of several weeks caused no difference in growth rate as compared with controls.

The solubility of the active material in phenol gives but little clue to its composition since some proteins and their derivatives and also nucleotides are soluble in this reagent. Phenol has been used as a solvent in the purification of antigens [Palmer and Gerlough, 1940; Morgan and Partridge, 1941], of anterior pituitary hormones [Freud, Laqueur, and Mühlbock, 1939], of the anti-anæmic principle of liver [Karrer, 1941], and of nucleotides [Warburg and Christian, 1938; Emmerie, 1938].

The properties of the purified material suggest the presence of protein and nucleic acid disintegration products which may be responsible, as nutrients, for the nucleoprotein synthesis. Similar large increases in N.P.P. have been found with protein breakdown products such as

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two different sources—pancreatin and snake venom—in producing the morphological changes described suggests that this enzyme may be one of the most active factors in the pancreatin fraction. The presence of other factors in the crude lecithinase preparations cannot, however, yet be excluded.

SUMMARY.

Material has been prepared from pancreatin which influences the growth of fresh explants of the 9-day chick embryo heart in roller-tubes. Cultures grown in a mixture of this material and embryo-extract show a much higher nucleoprotein phosphorus (N.P.P.) content than do control cultures grown in embryo-extract alone. They also have a characteristic dense compact appearance and are composed of well-nourished polyhedral cells with numerous mitotic figures.

A method for the partial purification of the factor (or factors) responsible for these phenomena is described. It appears that at least two components are involved, one of which is responsible for the characteristic morphological changes in the cells, while the other can produce even in the absence of embryo-juice a very marked rise in N.P.P., as great as that produced under similar conditions by a very concentrated extract of embryonic tissue. Such nucleoprotein synthesis appears to be a necessary preliminary to cell division in cultures stimulated by the factor. This would be in accordance with the views of Willmer [1942 b, 1943].

Some chemical properties of the purified material are described. It appears to consist mainly of polypeptides and nucleotide derivatives. Crude preparations of lecithinase A from pancreatin and snake venom produce the morphological changes described, and this enzyme may be one of the active components.

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A.

B.

C.

D.

E.

F.

A. Culture grown in embryo extract alone for test period of 48 hours   × 10 8.
B Culture grown in embryo extract + crude material from pancreatin for test
       period of 48 hours.   × 10 8.
C Section of culture grown in embryo-extract alone for test period of 24 hours
           × 224
D. Section of culture grown in embryo-extract + crude material from pancreatin
       for test period of 24 hours   × 224
E. Section of culture grown in Tyrode solution alone for test period of 24 hours.
       × 224
F. Section of culture grown in Tyrode solution + phenol soluble fraction
       (N = 25 mg. per 100 ml.) for test period of 24 hours.   × 224.

RUM CHOLINE ESTERASE IN BARBITURATE ADDICTION AND EPILEPSY. By F. Schütz. From the Medical School, University of Birmingham.

was previously reported that prolonged administration of barurates was followed by a pronounced decrease of serum-cholineerase activity in man [Schütz, 1941 and 1943 *a*]. It was shown it this was due to an actual decrease in the amount of enzyme present her than to the action of an inhibitor. The simplest explanation account for this fact seemed to be that under the influence of the rcotic the activity of the cholinergic system is reduced and that erefore less choline esterase is needed, which consequently slowly creases.

Since the barbiturates are known to be effective in reducing the umber of epileptic seizures, but cause, after sudden withdrawal, violent istinence symptoms with greatly increased frequency of fits, a closer udy of the choline esterase in epileptics during administration and after ithdrawal of the drug was the subject of the experiments described low.

The increase in the number of seizures after drug withdrawal, far ove the level characteristic for the particular case when under no eatment, was thought to represent a rare occasion where abstinence mptoms could be followed quantitatively. The peak in the number seizures soon after the drug is withheld may indeed be compared ith the great demand for the drug in cases of drug addiction. The umber of seizures, however, is a conveniently more measurable quantity an the expressed desire for the drug in the latter case. In the present ivestigation the choline esterase was therefore determined on the me patients at frequent intervals before and during administration, nd after withdrawal of the drug. The number and kind of seizures as recorded as well as the mental condition during that time.

METHOD.

Through the kindness of Dr. J. J. O'Reilly, Medical Superintendent of the Birmingham City Mental Hospital, certain cases of epilepsy under his care were chosen by him and tentatively taken off the barbiturate treatment. Since the sudden withdrawal of the drug is known

to increase the number of seizures considerably, only a comparatively small number of such cases could be found with which such an experiment could be ventured. Moreover, not all of the cases could be kept off the treatment for any length of time, since in some of them the abstinence symptoms became alarming, and treatment had to be restarted immediately. It was, however, possible to establish even in these cases both the choline esterase and the number of seizures during an abstinence period. In other cases the withdrawal of the drug was, though followed by an increase of seizures, more easily tolerated, and these cases could therefore be studied for a longer period after withdrawal (4–6 weeks), and thereafter again under the influence of the drug.

Blood was taken from the arm vein in the morning before breakfast. The syringes and needles were cleaned and boiled and used as dry as possible to avoid hæmolysis. Germicidal fluids, in which syringes and needles are often kept, were not used, since they contain potent inhibitors of choline esterase. One to two hours after the blood was taken, the clot was detached and the serum separated in the usual way after centrifuging. The activity of the sera was usually determined on the same day; sometimes this was postponed to the following day, the serum being kept in the refrigerator (2–4°). This delay was found not to influence the activity to any appreciable extent. The choline esterase activity of the sera was determined by a continuous titration method [Hall and Lucas, 1937] and expressed, as described previously [Schütz, 1943 a], by the number representing the initial slope of the titration curve [(vol. of 0·01 N NaOH in ml./min.) × 10], when 25 mg. acetylcholine chloride B.D.H. was acted upon by 0·5 ml. serum at pH 8·0 and 37° C.

RESULTS.

It was found that the relationship between the number of seizures and the level of the choline esterase could best be made visible by plotting both against time (see figures). The absolute scales of the ordinate (choline esterase and seizures/day) are, of course, different according to the individual levels found in different cases. In some cases the mental behaviour of the patients changed, so that a marked psychosis of short duration developed during the abstinence period and this is denoted in the figures by a line parallel to the abscissa.

All cases were diagnosed as suffering from cryptogenic (idiopathic) epilepsy. Most of them received twice daily a dose of 195 mg. of phenyl-methyl barbituric acid (methophenobarbital), if not otherwise mentioned. Some of them received one such dose in the morning and double this dose in the evening. Some of the patients had been treated with methyl-ethyl-phenyl barbituric acid (phemitone) about a year earlier.

The well-known effect of the withdrawal of the drug, which consists mainly in an increase in the number of seizures, far above the frequency the particular case ever had previous to treatment, was clearly seen in all cases. In the case of W. P. (fig. 1) the peak of the number of seizures follows fairly soon after the withdrawal of the drug. The dotted line represents a tentative interpolation drawn to make the peak of the seizures more clearly visible. After withdrawal of the
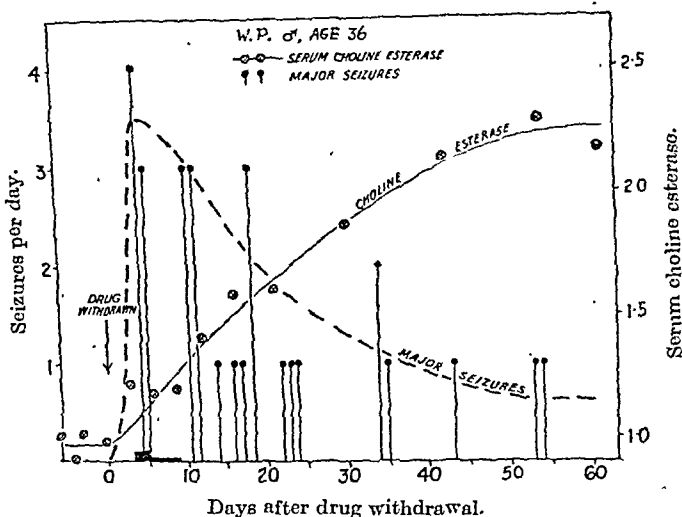
FIG. 1.—At the time of the peak of seizures after the withdrawal of the barbiturate the serum choline esterase had only recovered from approximately 40 to 45 per cent. of its final level. When the seizures were maximal the narcotic action of the drug had apparently worn off, whereas the choline esterase was still at the low level to which it had fallen during the treatment, and had only started its recovery. When 100 per cent. recovered there was no excess number of seizures, nor any other abstinence symptom.

drug the choline esterase activity rises slowly, but, as can be seen from figs. 1 and 2, it is still at a very low level at the time of the peak frequency of seizures. Later, while the seizures become less frequent again, the choline esterase rises more, until it reaches a level which is characteristic for the particular individual when without treatment; it should be noted that it reaches this highest level at very much the same time as the abstinence symptoms disappear again, i.e. when the frequency of seizures reaches a level which is constant and may be regarded as characteristic for the particular individual when under no treatment. After remaining 4–6 weeks without treatment, when both number of seizures and the choline esterase had reached a fairly constant level, treatment was started again. This caused an almost immediate fall in the number of seizures, whereas the choline esterase, though falling, reacted again much more slowly than the seizures. Three weeks after treatment had been restarted the choline esterase reached its

former low level. The choline esterase was still almost at its former high level at the same time as the fits had already fallen to what proved to be the lowest frequency-level which could be reached in this case. Another case, shown in fig. 2, exhibited major as well as petit mal seizures. Here the abstinence symptoms developed later than in the preceding case, but again the choline esterase was still found at a
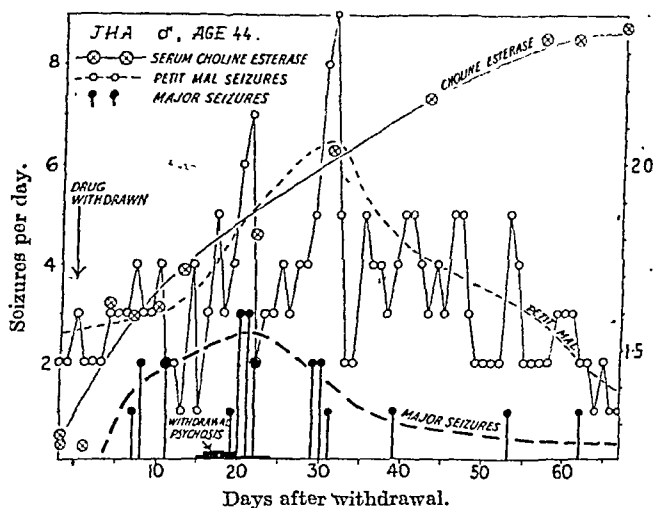
FIG. 2.—At the time of the peak of the withdrawal psychosis (thick line on abscissa) the serum choline-esterase had recovered from 35 to 55 per cent. *circa*; at the time of the peak of major seizures to 60 per cent., and to approximately 73 per cent. when the petit mal seizures were maximal. When 100 per cent. recovered there was no excess number of seizures, nor any other abstinence symptom.

relatively low level when the seizures had already reached their peak frequency.

If the level of the choline esterase, which was finally reached some weeks after the withdrawal of the drug, is taken as 100 per cent., prolonged treatment had reduced it previously to approximately 40 per cent. in the case of W. P. (fig. 1) and to 30 per cent. in the case of J. H. A. (fig. 2). At the time of the peak frequency of seizures it had only recovered from 40 to 44 per cent. in the first, and from 30 to 60 per cent. in the latter case. Two other cases gave results very similar to those just described. Both were men (25 and 28 years) and one exhibited grand mals only, the other grand and petit mals. When the seizures were at peak frequency during the abstinence period the choline esterase had only recovered from 28 to 38 per cent. in the first and from 45 to 58 per cent. in the second case. The estimation of the percentage reduction of choline esterase was made by means of a standard curve previously constructed, which related enzyme activity and quantity of unpurified human serum [Schütz, 1943 a].

In three further cases the abstinence symptoms developed so gravely that treatment had soon to be restarted. It is therefore impossible to state whether the "peak frequency" of seizures had actually been reached. In view of the other cases, however, it may be inferred that, since the number of seizures in these three cases was very high, the peak frequency could not well have been very much more than what was evident during the last day of abstinence. Similarly, the value of choline esterase for these patients, when under no treatment for at least three weeks, is also unknown. No absolute numbers are therefore available to show that the choline esterase had not recovered completely at the time of the peak frequency of seizures, as it was given for the first four cases. In the case of Miss E. L. R. (age 33 years) seizures rose to 8 on one day, eight days after withdrawal. Treatment was then restarted. During the earlier period of treatment she had an average of 2 seizures per month. When the 8 seizures appeared on one day the choline esterase of the blood taken in the morning was at practically the same low level as during the treatment. Evidently recovery of the enzyme had not started then. In the case of Mr. J. B. (age 32 years) seizures rose from an average of 10 per month during treatment, to 4, 5, 4, and 12 per day on the first, second, third, and fourth day respectively after withdrawal. Treatment was restarted on the fourth day after withdrawal. The average level of the choline esterase during treatment was just below 1·0. This rose to 1·2 on the third, and to 1·4 on the fifth day after withdrawal, which was actually one day after treatment was restarted. In this case the choline esterase had definitely risen above the level it maintained during the treatment, but it becomes very probable from the foregoing cases, as well as from the absolute values found in epileptics when under no treatment (see below), that the value reached during the last day of the abstinence period was by no means near to what may reasonably be expected to have been a full recovery of the choline esterase in this case.

In the case of C. C. (fig. 3) the number of seizures rose very sharply after withdrawal, and an obvious withdrawal psychosis developed. The choline esterase did rise soon after the withdrawal, and at a more rapid speed in this case than in the other cases. But also here it can be said, as for the two foregoing cases, that it seems most unlikely that the choline esterase had reached anything near a 100 per cent. recovery at the time of the great excess number of seizures.

In fig. 4 the choline esterase level in four non-epileptics is shown after a barbiturate treatment lasting four weeks, and after sudden withdrawal of the drug. It can be seen that the speed and extent of fall of choline esterase after onset, and its rise after withdrawal of the drug, was found to be essentially the same as in epileptics.
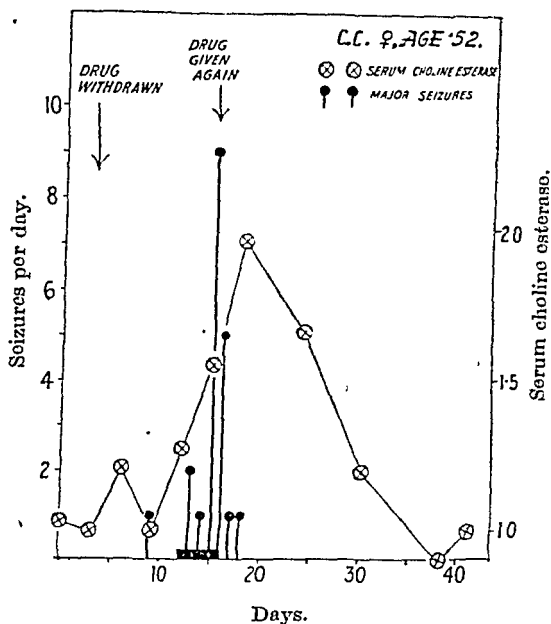
Fig. 3.—In this case the withdrawal symptoms were so intense that the barbiturate had to be given again. When the seizures were very frequent, the serum choline esterase, though considerably higher than during the treatment, had not yet recovered to what was the usual range of level in normals or epileptics.
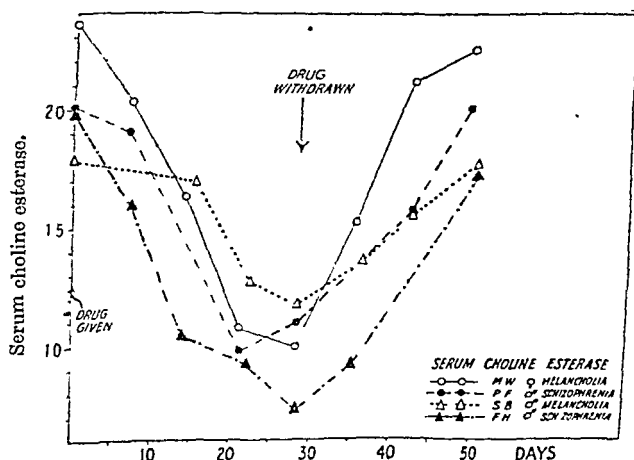
Fig. 4.—Four non-epileptics under the same barbiturate treatment as was given to the epileptics.   The serum choline esterase was lowered by the prolonged treatment, and rose after withdrawal of the drug at much the same rate as in epileptics.

Withdrawal Psychosis and Choline Esterase.

Besides the increasing number of seizures the mental behaviour of the patients offered another abstinence symptom. The thick line drawn parallel to the abscissa (see figures) denotes what clinicians described as "extreme confusion with occasional restlessness and noisiness," the thin line "mild confusion and restlessness." It can be seen that in the cases where such periods appear they coincide roughly with the period of the peak of seizures, or actually preceded the maximum of seizures a little.

This psychosis does not seem to be a secondary effect of the increased epileptic activity, as the more chronic mental changes in epileptics probably are. In one case (H. G., male, 38 years) it even represented the only sign of abstinence. This case had seizures for a few years and even went through a status epilepticus one year ago. At the time of the experiment he had had no fits for three months, and was under no treatment at all since seven weeks. He was put on barbiturate treatment again and no seizures occurred. When he had been under the treatment for three weeks and the choline esterase had fallen to the usual low level, i.e. from approximately 2·5 to 1·3, the drug was withdrawn, but even then no seizures occurred. However, he became very restless, noisy and extremely confused, and had to be confined to bed. The psychosis started three days after the drug was withdrawn and lasted one week. It was very similar to the state observed in those cases where it appeared together with seizures.

These mental abstinence symptoms showed a very similar relationship to the choline esterase as did the number of seizures in the other cases. When the confusion was worst the level of choline esterase was very low (1·4) compared with that found before treatment, or three weeks after withdrawal (2·5). When the withdrawal psychosis had disappeared the choline esterase was higher again (1·8) and had recovered approximately half-way towards the level which proved to be the highest level, and characteristic for the particular patient when under no treatment.

Time Sequence of Abstinence Symptoms.

Since the mental disturbance, which occurred during the abstinence period, lasted usually only a few days, a peak period of this psychosis was quite evident, similar to the peak periods of the grand and petit mals. There were a few cases in which these three peaks could all be recorded in the same individuals, and it was found that they did not appear at quite the same time, but showed a somewhat regular time sequence. The period of gross mental disturbance appeared first, followed by the peak of grand mal seizures, and finally the peak of the petit mals occurred. The psychosis had already improved

considerably when the grand mal seizures reached their peak number. Similarly, the grand mals had decreased, usually to about half their maximal frequency, when the petit mals were at their peak.

Only three cases exhibited all three abstinence symptoms. The time interval between the maximum of the mental·disturbance and the peak of grand mals was 1–3 days, that between the peaks of grand mals and petit mals was even greater still (3–7 days). Also in the other cases, which exhibited only two of the three peaks, the time sequence, as described above, was always observed.

Although the number of observations is small, brief mention of this observation was thought justifiable, because, if future investigators could substantiate it, it might provide an indication of whether different degrees of discrepancies between cholinergic activity and the actual level of choline esterase are a factor in producing the three different symptoms. In the cases mentioned, at any rate, the choline esterase was lowest during the withdrawal psychosis; during the peak of grand mal seizures it was higher, but still lower than during the peak period of the petit mals, which followed.

Atypical Case.

Among the cases studied one appeared atypical in a number of respects (fig. 5). An unusually high level of choline esterase was found, even when under the barbiturate treatment, and after withdrawal of the drug oscillations of the choline esterase were observed, not seen in any other cases. In one instance, however, the findings are in harmony with those made in the other cases. The choline esterase was lower after prolonged treatment, and rose after withdrawal of the drug. But the absolute height of choline esterase seems quite abnormal. This was, even during treatment, as high as 3.4. In more than twenty similar cases under the same prolonged treatment the choline esterase value was never higher than 1.2, with an average of 0.7 (S.D.M. ± 0.04). The values found in this case, when under treatment, are even higher than the average found in untreated normals $(2.1 \pm 0.14 \ (n = 16))$, or untreated epileptics $(2.4 \pm 0.23 \ (n = 13))$. When the drug was withdrawn the choline esterase did rise also in this case, but its rise was not smooth as in all other cases. It showed up and downward moves with two minor peaks, finally reaching the extraordinary high level of 4.7. Besides the fact that this was the highest value recorded in man in the course of more than 100 observations, so sudden a rise and fall of the choline esterase was never observed.

The occurrence of the abstinence symptoms, and their wearing off in relation to the choline esterase, seems, however, to be in harmony with the principle found in other cases. The symptoms had worn off when the choline esterase reached the highest level. It even seems

that the upward rise of the choline esterase was somewhat stopped during the peak period of seizures.

The most unusual aspect of this case, besides the extraordinary high absolute value of choline esterase, was the ease with which the abstinence period was overcome. While all other cases were during that time, apart from the great epileptic activity, in a rather grave state, exhausted, confused, etc., this patient did not show any particular
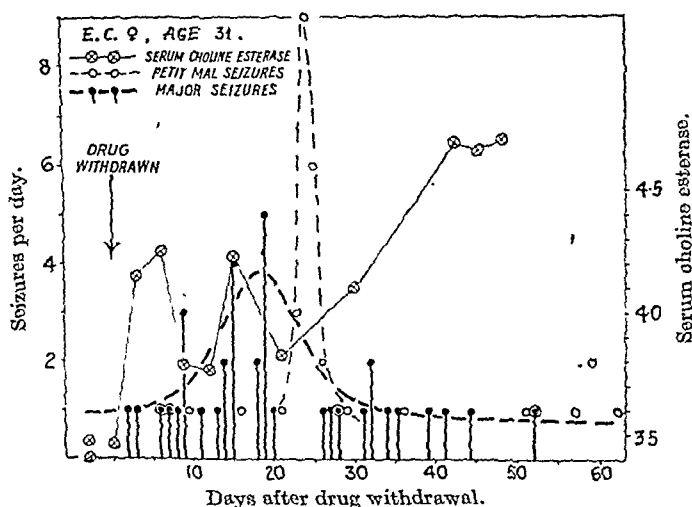
Fig. 5.—An atypical case. The level of the serum choline esterase is extraordinarily high, both during treatment and after withdrawal. Its initial recovery after withdrawal is much greater and quicker than in other cases. But, as in the other cases, the level of the choline esterase had not recovered completely when the seizures were maximal. When recovery was complete there was no excess number of seizures. The peak of the major seizures preceded the peak of petit mal seizures as in the other cases.

general change, and also the frequency of seizures decreased a little sooner than in the other cases.

Since a relatively low choline esterase was always found during the abstinence symptoms, and the rise of choline esterase was always found to go somewhat parallel with the reduction in the frequency of seizures, the very high value of choline esterase in this case might have something to do with the attenuated withdrawal symptoms.

No explanation can be advanced, of course, for the very high choline esterase levels themselves, as well as for their unusual upwards and downwards oscillations. Many factors influencing the level of choline esterase are, of course, still unknown, and it must be recognised that not all movements of the latter after withdrawal of the drug may be attributed to the withdrawal only.

Choline Esterase in Epileptics under no Treatment.

Since the great majority of epileptics are nowadays under some treatment, it was found difficult to obtain a sufficient number of untreated cases in order to establish whether the choline esterase level in epileptics is different from that in normals. There were a few cases available which had been under no treatment for some time, but these cases were mild ones and exhibited a low number of seizures only. The values found are shown in Table I, where also those cases are

TABLE I.—CHOLINE ESTERASE ACTIVITY OF SERA FROM EPILEPTICS UNDER NO TREATMENT FOR AT LEAST THREE WEEKS.

Activity = (vol. 0·01 N NaOH in ml./min.) × 10.

25 mg. acetylcholine chloride, 0·5 ml. serum, pH 8·0, 37° C.

No.	Choline esterase activity.
1	2·27
2	2·66
3	3·06
4	4·71
5	1·57
6	2·37
7	2·55
8	2·41
9	2·25
10	1·68
11	2·35
12	1·82
13	1·59
Average	2·44
S.D.M.	±0·23

included which were described above, three or more weeks after withdrawal of the barbiturate. The period of three weeks was previously found to be just sufficient to restore the choline esterase to what appeared to be the highest level characteristic for the particular patient.

In 20 observations on normals an average value of 2·07 (S.D.M. = ±0·15) was found. The average value found in epileptics under no treatment is a little higher (2·44±0·23) ($n=13$), but the difference can hardly be regarded as significant. The degrees of freedom of our experiment being 31, and $t=1·48$, the value of P, corresponding to these figures, was found in Fisher's tables (1935) to be 0·2, indicating that the probability of the observed difference being due to the error of selection is 1 : 5. The difference cannot, therefore, be regarded as significant.

It should, however, be noted that only mild cases of epilepsy were available, since the graver cases are mostly under treatment. It should, furthermore, be noted that the values found in some epileptics are much higher than anything obtained from normals, or non-epileptics. Since it is recognised that a number of different pathological states might still be embraced with the diagnosis of cryptogenic (idiopathic) epilepsy, further investigators may perhaps show whether there exists one group of epileptics showing a significantly higher choline esterase than others, or normals.

At present it can be stated that in an unselected group of rather mild cases of epilepsy the average value was not significantly higher than that of normals, with some individual cases, however, showing higher values than any observed hitherto in normals or non-epileptic patients.

DISCUSSION.

The number of experiments described in this paper is small, and the conclusions, therefore, are of a tentative nature. The number of experiments could not be enlarged, because of the well-founded reluctance of clinicians to withdraw the drug from epileptics. Another point which should affect the weight attributed to the observations is the fact that, owing to the untoward effects of the drug withdrawal, no really grave cases of epilepsy have been included in this study. It seems not impossible, therefore, that future investigators may find at least some of the features described in this paper more pronounced in their cases if they succeed in investigating graver cases of epilepsy on similar lines. On the other hand, it may be that what appeared in the small series described here to be an "atypical" behaviour (in one of 9 cases) might prove to be not so uncommon. Certain features of our results, however, appeared clearly, and since they are concerned with significant changes in the same individuals a conception of the underlying mechanism could easily be formed.

Mechanism of Withdrawal Symptoms.—The observations are summarised in fig. 6.

When the drug is given there is a reduction of both frequency of seizures and choline esterase (at *a*, fig. 6). The speed, however, with which these phenomena develop is greatly different. The fall in the number of seizures takes place almost immediately after treatment is started, the seizures reaching their lowest frequency when the choline esterase is still quite high. Similarly, after withdrawal of the drug, the frequency of the seizures rises much sooner than the choline esterase. Invariably the choline esterase was found still low when the frequency of seizures had already reached its maximum. At that time the choline esterase was either quite as low as during the narcotic treatment, or it was raised, but far from having reached what proved to be the

characteristic high level for the particular individual when under no treatment.

The reduction of the choline esterase activity of serum in man after prolonged treatment with barbiturates has previously been shown to be due to a diminution of the amount of enzyme present rather than to the action of an inhibitor [Schütz, 1934 a]. It seems justified, therefore, to interpret this fall of choline esterase as a counter adaptation: the activity of the cholinergic system being reduced by the narcotic resulting in a diminished output of acetylcholine, reducing
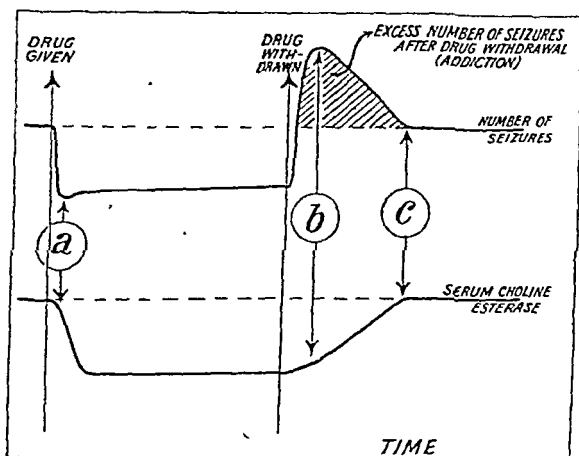
FIG. 6.—Relationship between frequency of epileptic seizures and serum choline esterase, after prolonged administration and abrupt withdrawal of barbiturates; see text. .

thereby the demand for choline esterase, which, consequently, slowly decreases. It seems that the level of the choline esterase may be regarded as an *indicator of the "average activity"* of the cholinergic system, and not dependent on temporary and transient changes of this activity. This view is supported by the fact that a large single dose of the narcotic is not followed by a fall of choline esterase, because it is apparently unable to cause a decrease of the average level of activity of the cholinergic system.

It is suggested that the excess number of seizures at *b* (fig. 6) is due to the fact that the narcotic effect of the drug, which diminishes the seizures, wears off more rapidly than the counter adaptation, namely, the reduction of the choline esterase. It should be noted that when the seizures reach their maximum (at *b*) the choline esterase is still low. The seizures, however, return to their "normal" number at about the same time that the choline esterase reaches its former level (at *c*).

On this theory, an optimal effect of the drug (greatest reduction

of seizures) should be expected at a, when the direct narcotic effect of the drug had obviously already taken place, but the counter adaptation (reduction of choline esterase) had not yet developed.   An observation, frequently made, seems to suggest that this is, indeed, the case. In the case of C. C., $e.g.$, an average of 2-4 fits per month could be recorded for years when the patient was under treatment.   When treatment was restarted after a period of abstinence, during which the choline esterase rose, no seizures occurred at all for the first month. In the second month there were 2 fits, and thereafter they occurred with much the same frequency as during the first period of treatment. During the second of these periods (after treatment was restarted) a higher choline esterase was present than during the later stages of the first period.   This point (drug tolerance) is, however, much less obvious than the relationships described for the periods after the withdrawal (addiction, b and c, fig. 6).   The fact that the first weeks of treatment always appeared to cause a slightly greater reduction of seizures than later on is shown in fig. 6.

To summarise the main points, it can be stated that at b there is little or no narcotic effect combined with a low choline esterase, whereas at c there is equally no narcotic effect but much less seizures, apparently owing to the high level of choline esterase which is then prevailing. These observations seem to offer a direct explanation for the abstinence syndrome in the case of barbiturates.

The literature on barbiturates has been carefully reviewed by Tatum [1939], the theories of drug addiction by Tatum and Seevers [1931] and recently by Adams [1937].   The explanations put forward in general terms to account for drug addiction (abstinence syndrome) are numerous.   Dixon [1930], for example, says "that the explanation for . . . withdrawal symptoms is that nerve cells, on reawakening, become hyperexcitable."   No conjectures are advanced on how this apparent "hyperexcitability of nerve cells" develops.

The experiments described above seem to make it unnecessary to assume any hyperexcitability of the nerve cells.   They suggest, however, that a status of hyperexcitation exists not because the cells are more excitable, but because of an inadequate amount of choline esterase present to deal with a fully active cholinergic system.   Indeed, the average level of excitability of the nerve cells may well be equal at b and c (fig. 6); but less regulated by the choline esterase at b than at c.

This explanation does not exclude, of course, the possibility, and indeed probability, that the choline esterase is not the only enzyme or enzyme-system deranged by prolonged administration of the drug. The choline esterase is, however, the first enzyme which has been shown to be related to abstinence symptoms.

$Cholinergic$ $System$ and $Convulsions.$—The results described in this

paper may also have implications in the theory of epilepsy and convulsions in general. It may be inferred that one important characteristic of the withdrawal status is what might be called a predominantly cholinergic status, brought about by the relative lack of choline esterase. The question arises, therefore, if such a status is a precipitating factor in epilepsy, since the great excess number of seizures could be observed during that period and disappeared when there was reason to believe that this hypercholinergic status had also disappeared, because of the adjustment of the choline esterase.

It could, of course, be argued that any sudden change in the life of an epileptic would almost certainly increase the frequency of seizures, and that a sudden withdrawal of a drug, or the status produced thereby, has nothing fundamental to do with the production of seizures. This argument can be dismissed because of the fact that convulsions, indistinguishable from major epileptic seizures, occur even in non-epileptics after the withdrawal of some drugs (barbiturates, alcohol, chloral, paraldehyde). It is well known, *e.g.*, that convulsions may *occur in cases of delirium tremens after withdrawal of alcohol in chronic* alcoholics. Recently Kalinowsky [1942] observed convulsions after withdrawal of soluble barbitone, which had been regularly given in large doses for one or two years. None of these patients' histories or family histories, nor (so far as they were obtained) their electroencephalograms, suggested epilepsy, and in no case has there been any recurrence of seizures since. Kalinowsky also found withdrawal *convulsions in non-epileptics with chloral and paraldehyde, but not* with opium or its derivatives. While chloral and paraldehyde are known to be effective in controlling seizures, opium is of little or no use in that respect. At any rate these convulsions are only seen after withdrawal in chronic drug addiction, they never occur in the acute intoxication caused by the same drugs. Kalinowsky does not suggest any possible mechanism involved in the production of seizures, but he *thinks that perhaps the same mechanisms are responsible for the* accompanying deliriums observed after alcohol and paraldehyde withdrawal.

Because of these facts the status prevailing during the abstinence period may be regarded as of more general importance for the production of seizures and not only a mere precipitating factor. A number of other facts seem to be directly explainable from the viewpoint that a predominantly cholinergic (hypervagal) status may be intimately connected with the production of seizures.

It has long been known, for example, that a certain group of epileptics have many more seizures during the night than during the day. Although little is known about the physiology of sleep, it is generally accepted that the cholinergic system is then at a relatively higher average level of activity than the adrenergic system (Hess):

and insoluble pigment were separately determined, from Groups I, III, IV, and from a similar unreported series with shorter survival time,
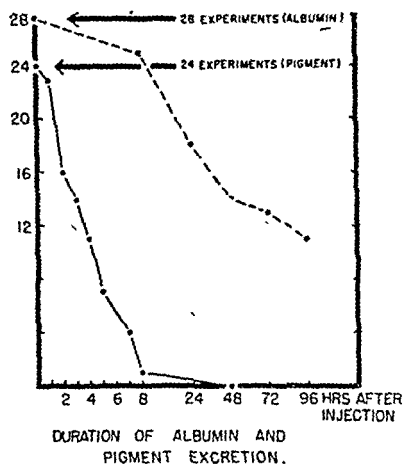
FIG. 9.—Duration of albuminuria (28 experiments) and pigment excretion (24 experiments), after injection.

shows that precipitation in the insoluble acid hæmatin form occurs in the more acid and more slowly secreted urines (Table IV).   While on the average the more swollen kidneys are found in those animals where the first urine contains 100–75 per cent. insoluble pigment, the Chi square test showed that this relation might occur three times out of ten on the basis of chance alone.

TABLE IV.—RELATIONSHIP OF PIGMENT PRECIPITATION TO URINE pH, FLOW, UREA CONCENTRATION, AND WEIGHT OF KIDNEYS.

Per cent. of total excreted pigment in deposit.	pH of urine.			Urine flow, ml./hr.			Urine urea, g. per cent.			Kidney weight, g./kg.		
	Frequency.		Mean.	Frequency.		Mean.	Frequency.		Mean.	Frequency.		Mean.
	Below 5·2.	5·2 or above.		Below 10.	10 or over.		Under 1.	1 or above.		Below 8.	8 or above.	
100–75	19	7	4·98	18	8	9·3	12	13	1·29	7	8	8·47
74–25	5	4	5·34	7	3	8·9	5	4	0·92	3	2	7·46
24–0	13	21	5·88	12	22	17·4	23	9	0·77	10	3	6·85
	$\chi^2 = 7·3$			$\chi^2 = 9·1$			$\chi^2 = 3·4$			$\chi^2 = 2·6$		

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between pH 6·4 and 5·8 met-hæmoglobin was formed, and that increasing the salt concentration produced a precipitate of acid hæmatin at a pH of 4·0 after incubation for an hour at body temperature. Our own analysis of urine pigment distribution in the soluble and insoluble phase bears out their contention. It must be pointed out, however, that the quantity of pigment used is very different. Yorke and Nauss had used about 25 g./kg. body wt. Baker and Dodds used multiple and large (but unspecified) amounts; De Gowin and his colleagues [1937] used single and multiple homologous hæmoglobin injections each of about 3–5 g./kg., as also did Wakeman *et al.* in dogs; De Navasquez in rabbits used single injections of about 400–500 mg./kg. and multiple injections totalling 600–2500 mg./kg. body wt. In man the usual minimum fatal dose of hæmoglobin is about 1 g./kg. body wt. (=500 ml. transfused blood). That is, most workers except De Navasquez, whose animals showed only a mild uræmia (in only one rabbit following a single injection did the blood urea reach 117 mg. per cent.), used doses larger than those ordinarily encountered in man.

Turning now to myoHb., the dosage used is very much smaller. We have produced uræmic death with amounts injected varying between 84 and 182 mg./kg. body wt. If this is compared with the findings in man (Table V) it may be seen that this range includes the calculated

TABLE V.—COMPARISON OF MYOHÆMOGLOBIN EXCRETION IN MAN AND IN THE RABBIT.

	Calculated dosage.	Amount excreted.	Max. plasma level.	Urine concentration.
	mg./kg. body-weight.		mg. per cent.	
MAN (Crush):				
(a) Fatal	175	7	..	335
(b) Recovery.	(?165)	165	..	400
RABBIT (Acid):				
(a) Fatal	182–84	60–0	..	265–0
(b) Severe uræmia .	240–50	121–0	150	1980–507
(c) Unaffected	180–50	172–0	158	875–0

value in a fatal human case of crush syndrome in which muscle pigment was measured. In a 40-kg. woman about 2 kg. of muscle were necrotic (50 c.c./kg. body wt.). MyoHb. in the dead muscle was 158 mg. per cent., compared with 510 mg. per cent. in the unaffected limb. Thus the total loss was 7 g. or 175 mg./kg. body wt. This patient excreted only 285 mg. Another 40-kg. (recovering) patient [Bywaters, 1943] excreted 6·6 g. of pigment: her urine was alkalinised, and it seems

*Group V (control).   Compression of Leg in Rabbits with Urine of
low pH on Acidifying Diet: no Pigment Injection.*

Five experiments showed no difference from the results seen in
rabbits with compression on a normal diet [Bywaters and Popjak, 1942]
except that the plasma CO_2 combining power and the pH of the urine
were low.   No increased rise in Hb. or blood urea was seen.   No
abnormality was seen in the kidneys at autopsy.

*Group VI (control).   Injection of Rabbit Muscle Extract into One Normal
Rabbit, One on Acid Diet, and Three on Acid Diet with restricted
Water Intake.*

These five rabbits, injected with amounts of protein between 97
and 380 mg./kg., excreted between 3·5 and 48 per cent. of the protein
injected, but showed negligible changes in urea or creatinine output,
or blood urea level.   The urine was protein-free by 21 hours (except
in one experiment).   Kidney weights at autopsy were normal, and no
naked-eye changes were seen.

DISCUSSION.

These experiments show conclusively that renal failure can be
induced in rabbits on an "acidifying" diet with urine of a pH below
6·0 following the injection of a solution containing human myoHb.
Renal failure does not develop after injection of this solution into
animals on a normal diet with urinary pH above this level.   The renal
failure was associated with large swollen kidneys which were seen as
early as 4 hours after injection.   The uræmic rabbits also showed,
besides a lower urinary pH, a slower rate of urine secretion and a
moderately high concentration of urinary urea in the first specimen
after injection.   The pigment was mostly in insoluble form.   By
contrast in rabbits which did not develop renal damage, fairly large
quantities of pigment were put out, the urine flow was faster, less
concentrated and less acid: the pigment was mostly in solution.   These
factors (rate of flow and concentration of urea in the first specimens)
seemed secondary in importance to the pH of the urine passed.

Thus these results confirm, for myoHb.-containing solutions, the
prime importance of the acidity of the urine first enunciated by Baker
and Dodds in 1925 for the "production of renal damage following
hæmoglobin injection."   Yorke and Nauss in 1911 had produced
oliguria and anuria following Hb. injection in the rabbit fed a dry
oats diet, but not in those fed green vegetables.   Baker and Dodds,
using rabbit hæmoglobin prepared by washing corpuscles with saline,
mechanical hæmolysis, and filtration, confirmed this effect of diet, and
showed that the essential point was the reaction of the urine.   *In vitro*
experiments with hæmoglobin in phosphate buffer solutions showed that

been the subject of further investigation, which will be detailed and discussed in relation to the work of other authors in a succeeding paper.

SUMMARY.

Following our failure to reproduce the renal effects of crush syndrome in rabbits, whose muscles are virtually devoid of myoHb., we have injected solutions containing this pigment into rabbits in doses equivalent to the amount excreted in human crushing injury.

1. In normal rabbits no lesion results.

2. If myoHb. is injected after release of a standardised leg compression, renal impairment may follow.

3. In rabbits with acid urine (pH 4·5–6·1) the injection of myoHb. solutions led to more severe renal failure, with death in uræmia in 4/25 cases. This renal damage was associated with pigment retention to a significant degree.

4. Experiments with limb compression and rabbit (non-pigmented) muscle extract injections in "acidified" rabbits gave negative results.

5. It is concluded, by analogy with these experiments, that myohæmoglobin excreted in an acid urine such as occurs in the crush syndrome may play an important rôle in the genesis of the renal failure seen in that type of injury. Analogies with the mismatched transfusion kidney are pointed out.

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probable that most of the released pigment was therefore excreted (and was in soluble form). This gives a value of 165 mg./kg. body wt. corresponding, on the same basis of pigment decrease in muscle, to 47 ml./kg. of muscle damaged. One reason for this difference in effective dosage between Hb. and myoHb. is undoubtedly the difference in molecular size. MyoHb. has a molecular weight of 17,500, compared with 68,000 for hæmoglobin: it thus passes through much smaller pores and is filtered through the glomerular membrane of the dog at 75 per cent. of the creatinine rate, and at 25 times the rate of hæmoglobin filtration. The threshold value, about 20 mg. per 100 c.c., is one-fifth that of hæmoglobin [Yuile and Clark, 1941], accounting for its more rapid and complete plasma clearance.

Finally, it is necessary to discuss whether the use of human myoHb. in the rabbit may introduce a fallacy into the argument, and whether the pigment or the other protein impurity in the solution used was responsible for the renal damage. It is impossible to dispose of the first objection entirely from our experiments, as myoHb. cannot be extracted from the few small muscles of the rabbit that contain it in quantities adequate for injection. Experiments by Winton and his colleagues (personal communication) indicate that renal damage may result in "acidified" dogs from the injection of dog myoHb. A heterologous reaction conditioned by pH of the urine would certainly be unusual; moreover, the striking difference between the results in acid and alkali-excreting rabbits corresponds so closely with the results found in animals using homologous hæmoglobin that it is difficult for us to believe that heterologous reactions play any part.

The second objection, that this picture may be due to the protein impurity rather than the myoHb. of the solution, is unfortunately also difficult to meet. We have been able to crystallise human myoHb., but have found it extremely difficult to separate this small quantity of crystal from the amorphous protein precipitate which comes down with it, beyond about 50 per cent. protein impurity. This objection seems never to have been raised with reference to hæmoglobin injections, and none of the authors cited have supplied nitrogen analyses of the hæmoglobin solutions they used. However, if the impurity can only be separated in vitro with such difficulty, it is improbable that, following necrosis of muscle, pigment could be differentially held back by dead cell membranes. A partial answer is given by the negative results of injection of rabbit muscle extract (Group V): no renal damage ensued either in normal acid or acid-dehydrated animals, despite an injected quantity of non-pigmented protein more than double that used in the pigment experiments.

The method by which myoHb. and Hb. solutions produce renal failure is by no means clear. This mechanism, and in particular whether retention of pigment is the result or the cause of renal damage, has

ON THE METABOLISM OF HISTAMINE. By Frank Alexander.

From the Department of Pharmacology, University of Edinburgh.

THE metabolism of histamine has probably received less attention than any other aspect of the pharmacology of this most interesting substance. Leiter [1925] studied the metabolism of several iminazole compounds but did not include histamine. He found that the body was able to destroy many of these compounds, although iminazole itself was excreted almost completely in the urine. Dale and Laidlaw [1910] and Oehme [1913] were unable to show any excretion of histamine in the urine even after the administration of quite large doses, and in 1930 Best and McHenry showed the natural occurrence of an enzyme system capable of destroying histamine. However, MacGregor and Peat [1933] found that histamine was excreted in the urine from a kidney perfused with blood containing a high concentration of histamine. Rose and Browne [1938] studied the clearance of histamine from various tissues of the rat after large doses had been given intravenously. They did not show any excretion of histamine in the urine. The observations of Kapeller-Adler [1941 *a*, 1941 *b*] on the excretion of histamine in the urine of patients with certain pathological conditions must renew interest in this aspect of its metabolism. The object of the present investigation was to study the clearance and excretion of histamine.

METHOD.

Histamine was estimated by the method of Barsoum and Gaddum [1935] as modified by Code [1937]. The tissue was minced and extracted by grinding with 10 per cent. trichloroacetic acid in the proportion of 15 ml. of acid to 10 g. tissue. The mixture was filtered by gravity and an aliquot of the filtrate taken. This avoided the tedious vacuum filtration and washing of the protein precipitate described in the original method. The aliquot was heated on a boiling-water bath with 10 ml. of concentrated hydrochloric acid for 1·5 hours. The procedure was then as described in the original papers. Urine was treated in a similar manner, 20 ml. being heated with 10 ml. of hydrochloric acid, and thereafter the procedure was the same as for the tissue filtrate. Parallel quantitative assays on guinea-pig's intestine and cat's blood pressure were carried out with all the different kinds of extract except those of

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subjected to the method of assay described, both before and after the addition of known quantities of histamine to the mince.　The results obtained are shown in Table I.

TABLE II.—HISTAMINE CONTENT OF NORMAL MICE.

Amount in body, μg.	Percentage in		
	Skin.	Gut.	Rest of body.
190	72·0	4·0	— 24·0
290	60·0	3·0	37·0
330	65·0	3·0	32·0
410	69·0	2·0	29·0

Table II shows the histamine content of normal mice and the distribution between certain tissues.　The distribution of intravenously injected histamine between the same tissues at various intervals after the injection is shown in Table III.

The clearance of histamine from the body and the relationship between the amount in the body and the amount destroyed or excreted
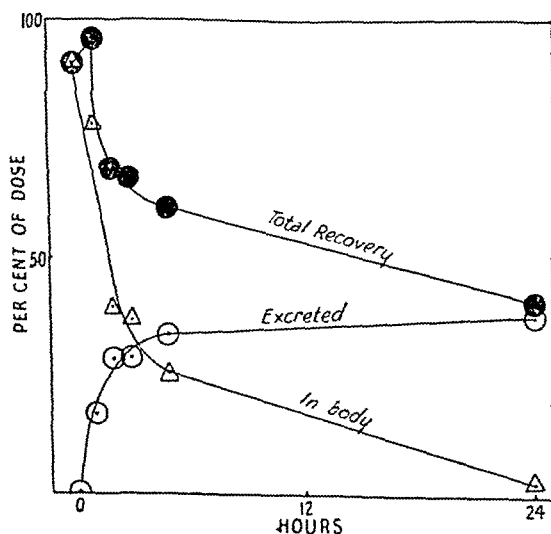
FIG. 1.—Amounts of injected histamine recovered from the body and excreta at different intervals after the injection.

is shown graphically in fig. 1.　The results were obtained by subtracting the mean normal histamine content of the mouse from the amount found after the injection.

sheep's blood without added histamine, and the evidence obtained in this way supported the view that the tests were actually measuring the concentration of histamine. The additional confirmatory test of desensitising a guinea-pig's gut to histamine by adding a very large dose to the bath as described by Barsoum and Gaddum [1935] was also made, the results of this test supported the evidence that the substance tested was histamine.

Sodium pentobarbitone was found to be a very satisfactory anæsthetic for the experiments on cats.

Normal mice were killed by a blow on the head, divided into three parts—the entire skin, the whole alimentary tract, and the rest of the body—and the histamine content of each fraction was determined. A series of similar mice were injected intravenously with a solution of histamine, each animal receiving 3·0 mg., a dose large enough to reduce the error due to variation in the histamine content of individual mice. The normal histamine content of a mouse is about 0·2–0·4 mg., and variations of this have a negligible effect on the result. Mice are particularly convenient for experiments of this kind, as with many other animals the amount of histamine in the body is more than a lethal dose. These injected mice were placed in individual metabolism cages, killed at intervals after the injection, their excreta collected, and the amount of histamine in the whole mouse and its excreta determined.

RESULTS.

To determine the accuracy of the method of estimation control experiments were conducted. Various tissues were minced and

TABLE I.—RECOVERIES OF KNOWN QUANTITIES OF HISTAMINE FROM TISSUES.

Tissue.	Histamine concentration, μg./gm.				Per cent. error.
	Initial.	Final.	Difference.	Added.	
Minced mouse	6·0	11·6	5·6	5·0	12
	7·0	17·7	10·7	10·0	7
	6·0	17·0	11·0	10·0	10
Minced liver	1·4	2·5	1·1	1·0	10
	1·4	2·5	1·1	1·0	10
Sheep's blood	0·10	1·14	1·04	1·0	4
	0·12	1·15	1·03	1·0	3
	0·11	1·20	1·09	1·0	9
				Mean .	7·3

experiments showed the excretion of a large amount of histamine in the urine and comparatively little in the fæces (Table IV).

<p align="center">TABLE IV.—EXCRETION OF INJECTED HISTAMINE.</p>

Mouse No.	Dose, mg.	Per cent. excreted.		Time, hours.
		Fæces.	Urine.	
1	3·0	0·4	'18·6	24
2	3·0	0·8	7·0	24
3	3·0	3·0	43·7	24
4	3·0	7·0	71·1	24
5	3·0	2·3	24·2	24
6	3·0	1·5	84·6	24
7	3·0	3·6	47·8	24
8	3·0	0·3	4·6	24
	Mean .	2·3	37·7	

DISCUSSION.

The experiments described showed that more than 60 per cent. of all the histamine in the body was contained by the skin. Although Harris [1927] found a high concentration of histamine in this tissue it probably was not thought to represent such a large part of the total amount present in the body.

The observations on the clearance of a large dose of histamine from the body showed the operations of at least two mechanisms. When a high concentration was present in the body a considerable amount was excreted in the urine, but when the concentration in the body fell below a certain level excretion in the urine ceased and the remaining part of the dose was removed by a different mechanism, such as the enzyme system described by Best and McHenry [1930]. Twenty-four hours after the administration the histamine content of the mice had reached its normal value.

Some interest is attached to the excretion of histamine in the urine. The experiments described here clearly showed that whilst the concentration of histamine in the body was high, excretion took place entirely through the kidneys. There was no evidence of destruction in the first hour after administration.

These observations are of interest when considered in conjunction with the finding of Kapeller-Adler [1941 a, 1941 b] that the urine in certain pathological conditions contained large quantities of histamine. In the experiments on mice histamine only appeared in the urine when there was a large excess in the body. If the results of Kapeller-Adler

Table III shows the distribution of histamine at intervals after its intravenous administration.

TABLE III.—DISTRIBUTION OF INJECTED HISTAMINE IN MICE.

Time, hours.	Percentage of dose present.		
	Skin.	Gut.	Rest of body.
0	4·7	26·4	52·8
0	7·2	8·1	85·1
0	2·8	6·0	87·5
0	7·5	6·3	68·1
1	0·0	7·5	81·4
1	7·9	41·3	39·0
1	13·1	9·1	58·8
1	7·2	6·3	44·0
2	9·4	6·4	31·1
2	13·7	6·8	22·5
2	17·8	14·3	5·0
2	2·1	6·3	10·9
3	22·0	20·0	32·4
3	11·3	12·8	50·8
3	0·0	6·4	11·8
3	1·3	9·8	30·8
5	1·7	12·3	15·6
5	0·7	8·6	15·1
5	8·5	5·7	24·7
5	0·5	6·0	11·2
24	0·0	0·1	0·0
24	0·0	0·0	0·0
24	1·0	1·3	9·4
24	0·0	0·6	0·4
24	0·7	0·3	2·0

In view of the failure of previous workers to show the presence of parenterally administered histamine in the urine, the observation of the excretion of large amounts by this route was studied more closely. The histamine excretion of normal mice was found to be very small. The amounts in the combined urine and fæces of 6 normal mice were estimated as 1·0, 1·2, 1·5, 2·0, 3·0, and 3·5 μg. of histamine per 24 hours. A metabolism cage was constructed whereby the fæces and urine of an individual mouse could be obtained separately, and the histamine excreted both by kidneys and alimentary tract was determined. Chloroform was placed in the collecting vessels to prevent bacterial growth, but it was doubtful whether this precaution was necessary. These

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These observations are of interest when considered in conjunction with the finding of Kapeller-Adler [1941 a, 1941 b] that the urine in certain pathological conditions contained large quantities of histamine. In the experiments on mice histamine only appeared in the urine when there was a large excess in the body. If the results of Kapeller-Adler

were due to an excess of histamine in the body, this excess must have been a large one.

SUMMARY.

The skin of the mice was found to contain more than 60 per cent. of all the histamine in the body.

A large dose of histamine was cleared partly by excretion and partly by some other mechanism. Excretion occurred through the kidney, and only whilst a high concentration was present in the body. The whole of a large dose was removed within twenty-four hours of administration.

ACKNOWLEDGMENTS.

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the pupils are small; the heart-rate slow, and the intestine active. The occurrence of a greater number of seizures in epileptics during sleep is, from the viewpoint outlined above, not surprising.

Another observation made in the cases described above may be mentioned here in connection with the question of the diurnal-nocturnal distribution of seizures. It was clearly observed that all cases appeared to be in a drowsy and sleepy state during the abstinence period. This was quite evident, even if occasionally they were noisy or restless. Even during these outbursts their state resembled rather one of sleepiness than of wakefulness. This seems all the more significant since that observation was made after the withdrawal of a narcotic. Thus a paradox presented itself: during administration of the narcotic (if made over prolonged periods of time) the patient appears more soundly awake, whereas after withdrawal of the narcotic he appears sleepy.

Besides this sleepy state other symptoms of the postulated predominantly cholinergic status were sought for, and indeed in all patients very small pupils were found, most of them of pin-point size, and the heart-rate was slow (50–65 per min.).

Why a hypercholinergic status should be a factor in the production of convulsions is not known. The findings of Hopkins [1935] could not ascertain that any of the "known changes in the acid-base equilibrium, water shifting, and other ionic blood changes" which he investigated can be made responsible for the increased number of seizures during sleep, since he found these changes greatest at one period of the night and that they did not show any parallelism to the peak incidence of seizures reported by other workers.

Some important discoveries made by Grinker [1938] and Penfield [1941] seem to point in the same direction as the interpretation of our results given above. By means of a number of electrodes placed on different parts of the skull, and through the nose against the sphenoid, these authors recorded the electro-encephalograms simultaneously as obtained from these different leads in the same individual. Penfield [1941] describes one case where, during the prodrome symptoms of an attack ("aura"), when the patient "felt like the start of a spell," no cortical abnormality could be detected, large waves, however, were found originating from the base (diencephalic?). The cortex only became involved later when the major attack developed. Since it is well known that the basal regions of the brain are closely connected with cholinergic activities and sleep, these discoveries seem greatly to support the conception derived from our results. These basal regions were also found to contain much greater quantities of choline esterase than the rest of the brain [Nachmansohn, 1938 and 1939]. Adam, McKail, Obrador, and Wilson [1939] found that stimulation of the hypothalamus in cats often as much as doubled the acetylcholine content of the cerebrospinal fluid. It would seem desirable to supple-

ment these electro-encephalographic localisations by a chemical approach such as determinations of the choline esterase in different brain regions in epileptics and non-epileptics.

Other findings pointing in the same direction were reported by Williams [1941] and Russell and Williams [1941]. An increase of petit mal activity by injection of several anticholine esterase substances was found by these authors. This effect was inhibited by atropine. Intravenous injection of acetylcholine was followed by an epileptic outburst on four occasions, with clinical evidence in two subjects, and was prevented by atropine.

The well-known precipitating factor of alcohol in epilepsy, for which no satisfactory explanation has been forwarded yet, is probably also connected with the cholinergic system, since Bader and Schütz [1943] have recently found that such low concentrations of alcohol, as can be expected to exist in or on nerve cells in an alcoholic state, destroy appreciable amounts of choline esterase *in vitro*.

If in epilepsy, or in a certain group of epileptics at any rate, this predominantly cholinergic state exists and, as so many facts suggest, it plays an important rôle in the production of seizures, an adequate way to counteract this state would be the administration of choline esterase—work in that direction is now being carried out.

In conclusion it may be said that during the withdrawal period, after prolonged administration of slow-acting barbiturates, a predominantly cholinergic status apparently exists because of a relative lack of choline esterase. Since convulsions occur during that state even in non-epileptics, this state is believed to play an important rôle in the production of seizures. Such diverse facts as the nocturnal peak incidence of seizures, the precipitating action of alcohol in epilepsy, the first appearance of abnormal electro-encephalograms at the basal regions of the brain in some cases, can all be understood more clearly by the assumption that the cholinergic system, or a part of it, is at a relatively high average level of activity and represents an important epileptogenic factor.

SUMMARY.

1. The previously described decrease of serum choline esterase after prolonged administration of barbiturates in man was studied in epileptics after sudden withdrawal of the drug. The number of seizures then rises sharply to a maximum, while the choline esterase is still at a low level.

2. The seizures become less frequent again when the choline esterase starts to rise, and usually disappear completely when the choline esterase has just reached what proves to be, in the particular individual, its highest level.

3. When the drug is given, the choline esterase decreases to a

minimum in about three weeks, whereas the number of seizures is minimal in a few days.

4. A time sequence of the abstinence symptoms was noted, the withdrawal psychosis appearing first and wearing off when the grand mals were at their peak, whereas the latter preceded the maximal frequency of petit mal seizures.

5. In an unselected group of cryptogenic (idiopathic) epileptics, when under no treatment for some time, a slightly higher average value of serum choline esterase was found than the normal average, the difference being statistically not significant. In some of the epileptics, however, much higher values were found than were ever found in normals. The possible existence of one group of epileptics, characterised by a high choline esterase, is discussed.

6. After the withdrawal of the drug the narcotic effect seems to wear off more rapidly than the choline esterase increases. Thus the cholinergic system, free from narcotic and probably at a high level of activity, is left with an inadequate amount of choline esterase. This discrepancy probably accounts for the abstinence symptoms.

7. Because of the relative lack of choline esterase during the period of abstinence symptoms, excessive cholinergic activity is assumed to exist. A number of indications for this are found, and its possible rôle in the production of seizures is discussed. Some diverse features, such as the peak incidence of seizures during the night, the precipitating action of alcohol in epilepsy, and some features in the electro-encephalograms, become more understandable from this viewpoint.

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THE PRODUCTION OF RENAL FAILURE FOLLOWING INJECTION OF SOLUTIONS CONTAINING MYOHÆMOGLOBIN.

By E. G. L. Bywaters [1] and J. K. Stead. From the Departments of Medicine and Pathology, British Postgraduate Medical School.

A preceding paper [Bywaters and Popjak, 1942] has described the production of "shock" in rabbits following compression of leg muscles by rubber tubing for a standard time of 4–6 hours: after release of the compression, plasma leaks into the area of ischæmic necrosis, which becomes œdematous: hæmoconcentration and a fall of blood pressure result from this plasma depletion. Besides these effects, changes were seen due to passage of substances from the injured part into the blood-stream. A comparison was drawn between this experimental lesion and that of the "crush syndrome" in man. The latter is the sequence of events following crushing or compression of the limb beneath debris for several hours [Bywaters and Beall, 1941]. Plasma is lost into the ischæmic area with consequent hæmoconcentration and, sometimes, fall in blood pressure: the affected limb becomes œdematous. All these effects were reproduced in the rabbit, as well as such features as acidosis and creatinuria due to diffusion from the necrotic muscle back into the blood-stream. Two features of the crush syndrome in man, however, were not seen in the animal experiments: firstly, no myohæmoglobin (myoHb.) was excreted by the rabbit; and, secondly, no renal failure developed. A rise in blood urea occurred, but this was small, short-lived, and associated with an increased rather than a decreased urinary output of urea. The kidneys showed no gross damage. In man, by contrast, myoHb. is excreted [Bywaters, Delory, Rimington, and Smiles, 1941]: renal impairment often develops, leading in two-thirds of the observed cases to death from uræmia [Bywaters, 1942].

The kidney damage of the crush syndrome in man is strikingly similar, both functionally and pathologically, to that sometimes seen following intravascular hæmolysis. This renal damage following hæmo-globinuria was experimentally produced in the rabbit by Yorke and Nauss in 1911; it has been investigated since by many workers, notably by Baker and Dodds [1925], who proved conclusively that uræmia was

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produced only if the animal was secreting an acid urine. They showed *in vitro* that hæmoglobin in solution is changed to met-hæmoglobin and acid hæmatin if the acidity and the salt concentration is high. These findings have been confirmed by other workers using other species [*e.g.* De Gowin and his colleagues in the dog, 1937]. Baker and Dodds' hypothesis, however, that renal damage is due chiefly to simple mechanical blockage by these relatively insoluble derivatives has not been universally accepted [*e.g.* De Gowin *et al.*, 1938; De Navasquez, 1940].

It occurred to us, therefore, that our failure to produce renal damage by ischæmic compression of the rabbit's limb might be due (as the absence of myohæmoglobinuria certainly is) to the virtual absence of myoHb. from the thigh muscles of the rabbit. To test this hypothesis, we have supplied this deficiency of muscle pigment by injecting solutions containing human myoHb. The results show that gross impairment of renal function results if the urine is acid.

METHODS.

Most of the experimental conditions and biochemical methods used were the same as have been described in the earlier paper [Bywaters and Popjak, 1942]; in addition:

(1) *Myohæmoglobin* was prepared from human muscle, using a modification of Theorell's [1932] method. In well-developed bodies without intravascular clotting (chiefly traffic accident deaths) the femoral artery was perfused with saline (up to 5 l.) until the venous return, assisted by massage and movement of the leg, was clear. The muscle, freed from fat, etc., was minced, ground with sand, and extracted with an equal volume of saline overnight in the refrigerator. After centrifuging, the proteins were precipitated at room temperature with one-fourth volume saturated basic lead acetate, excess lead being removed with saturated disodium phosphate until no further precipitate occurred. Traces of other protein were removed by half saturation with ammonium sulphate and the myoHb. precipitated by full saturation. The myoHb. was then redissolved in distilled water and dialysed against running tap water, centrifuged, and the supernatant fluid finally concentrated by pervaporation at a temperature of $0°$ C. to a strength of about 1 g. per cent. Sodium chloride was added to isotonic strength, the solution sterilised by Seitz filtration and stored in the refrigerator. The yield was about 130 mg. per 100 g. wet muscle (estimated as hæmoglobin). 300–400 mg. per cent. of pigment was lost in the process of purification, since we have found human muscle to contain about 500 mg. In some samples faint met-myoHb. bands were seen. These myoHb. solutions gave a nitrogen content (micro-Kjeldahl) varying between 2·18 and 2·40 times the theoretical value (17·35 g. N_2 per 100 g.

pigment as Hb.), calculated from the pigment content. Thus, an approximately equal quantity of non-pigmented protein was injected each time with the myoHb.

These pigment solutions could be given, even as fast as 25 ml. in two minutes, without any untoward reaction. (Some earlier preparations had induced immediate rigors (three experiments) or convulsions (three experiments), leading in two other animals, not included in the series, to death at 10 and 36 minutes respectively with dilatation of the right heart due to obstruction in the pulmonary circuit. Centrifuging the myoHb. solution immediately before injection eliminated these reactions by removing a very fine white precipitate, probably denatured protein, which sometimes appeared.) The usual rate of injection was 5 ml./min.: a few experiments where the solution was injected over a longer period (*e.g.* 27 min.) showed no consistent difference in results.

(2) Control muscle extract was prepared from normal perfused rabbit muscle, containing no myoHb., and extracted by exactly the same process. This was nearly colourless, showed no bands on spectroscopic examination, and was adjusted for injection to have a protein nitrogen content ranging from 164 to 610 mg. per cent.

(3) MyoHb. was estimated in the injection fluid and urine as "mg. hæmoglobin" by comparison of its alkaline hæmatin with that of a known alkaline hæmatin prepared from hæmoglobin [Clegg and King, 1942]. Pigment was estimated in the plasma by the benzidine method [Bing and Baker, 1932], the position of the band being read in the Hartridge reversion spectroscope. Care had to be exercised in taking blood from the ear vein that no hæmolysis occurred. A free flow is essential, and the blood collected into plain centrifuge tubes was spun immediately so that the serum could be removed before clotting occurred.

(4) Acidosis was induced by moistening the dry food each day with 15–20 ml. of 5 g. per cent. ammonium chloride, made up to 200 ml. with tap water. On this regime the urinary pH ranged between 4·5 and 6·0, averaging 5·0. The serum showed a CO_2 combining power sometimes as low as 10 vol. per cent., averaging 21 vol. per cent. Over the pre-injection control period of 4 to 14 days no ill-effects were seen from this diet except in 2 out of 35 "acid" experiments. In the one the animal had an unexplained temporary collapse, and in the other, the fluid intake of the animal being restricted, traces of albumen appeared in the urine.

(5) While in most experiments the rabbits were allowed free access to water, in some the fluid intake was restricted to 100 ml. water daily, which was used to moisten the dry food. The control blood urea readings were rather higher, averaging about 40 mg. per cent., than in the unrestricted water group, in which the average was about 30 mg. per cent.

produced only if the animal was secreting an acid urine. They showed *in vitro* that hæmoglobin in solution is changed to met-hæmoglobin and acid hæmatin if the acidity and the salt concentration is high. These findings have been confirmed by other workers using other species [*e.g.* De Gowin and his colleagues in the dog, 1937]. Baker and Dodds' hypothesis, however, that renal damage is due chiefly to simple mechanical blockage by these relatively insoluble derivatives has not been universally accepted [*e.g.* De Gowin *et al.*, 1938; De Navasquez, 1940].

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injected) except with the smallest doses.   The excretion of albumen stops simultaneously with pigment excretion except where on occasion a small amount (10 mg. per cent.) is seen in the next 24-hr. specimen. Kidney weights at death were within the normal range (*i.e.* $6 \cdot 0 \pm$ St. Dev. $0 \cdot 9$ g./kg. in 20 normal rabbits).   A similar result was seen if the fluid intake was restricted.

Group II.   Myohæmoglobin Injection in Rabbits following Release of Leg Compression.

When myoHb. (45–200 mg./kg.) was injected after release of a standard leg compression, a very much higher rise in blood urea was
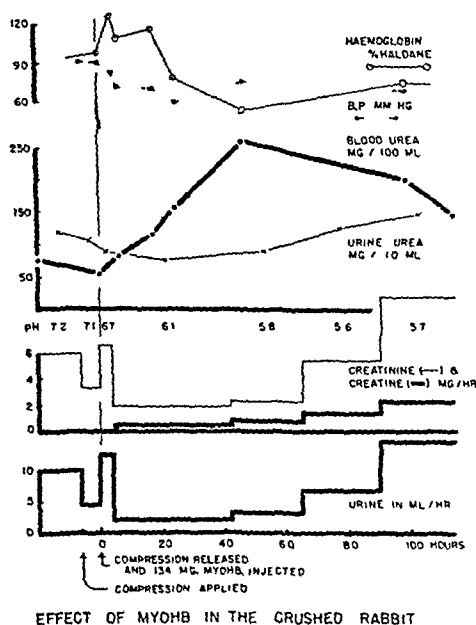
EFFECT OF MYOHB IN THE CRUSHED RABBIT

Fig 2.—Following release of leg compression, injection of 45 mg. myoHb. per kilo body-weight.   Note oliguria with nitrogen retention and depressed urinary concentration.

produced in 2 out of 6 experiments than in any rabbit subjected to the standard compression alone [Bywaters and Popjak, 1942].   This rise of blood urea was accompanied by oliguria, decreased creatinine output, delay in the usual post-compression creatinuria, and a depression of urinary urea concentration pointing to renal dysfunction in contrast to the results of standard compression alone.   There was also seen, following the initial hæmoconcentration and fall of blood pressure, a high degree of hæmodilution with a peak corresponding to that of the blood urea (fig. 2).   Recovery occurred with the passage of large pigmented casts.   Four other animals, however, showed a rise of blood urea no

(6) Significance of results was evaluated, using Yate's modification of the Chi square test for fourfold tables.

<div align="center">RESULTS.</div>

Group I.   Injection of Myohæmoglobin into Normal Rabbits.

In 8 experiments, where normal rabbits were allowed to survive for at least 28 hr. after injection, myoHb. was given intravenously in amounts ranging between 34 and 213 mg./kg. body-weight; no significant rise in blood urea occurred. Serum myoHb., 40 mg. per cent. after injections of 50 mg./kg., had disappeared by 1 hr. The urine. (pH 6·7–7·2) contained myoHb. in solution, often with met-myoHb.

EFFECTS OF MYOHB IN NORMAL RABBIT
(1660 G)
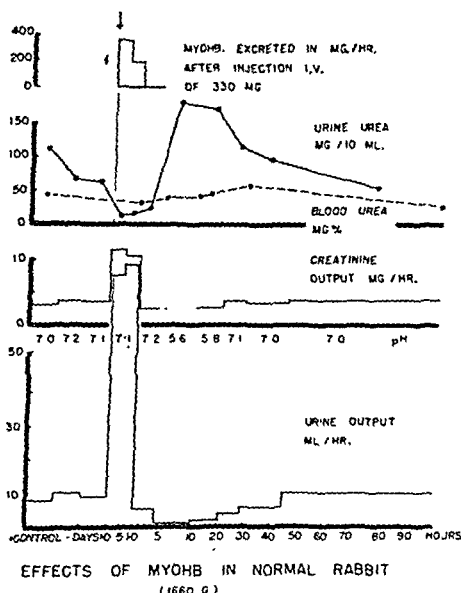
FIG. 1.—Normal diet: injection of 200 mg. myoHb. per kilo body-weight. Note diuresis and consequent oliguria: no renal impairment. 98 per cent. excreted in 64 minutes, all in solution.

bands faintly visible, but no insoluble pigment except a rare granule or cast seen microscopically. As may be seen from fig. 1, depicting a typical experiment, a diuresis usually follows the injection, with a resultant depression in urinary urea and creatinine concentration: there is usually during this first period, however, an increase in the amount of urea and creatinine excreted in unit time, and this is followed either by a return to normal if the diuresis is small or, if large, by an oliguric period (fig. 1) with increased urea and creatinine urinary concentrations and decreased total output of these substances. Over the whole period there is little change in clearance. A very small proportion of the pigment is retained (e.g. 1, 2, 4, and 12 per cent. of that
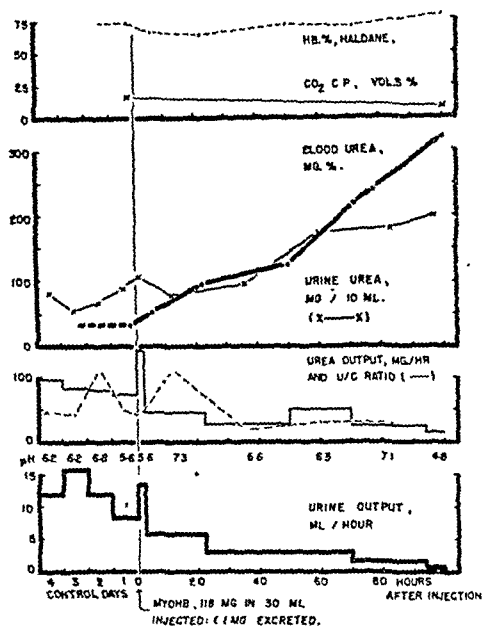
EFFECT OF MYOHB IN THE "ACID" RABBIT.

Fig. 3.—"Acid" rabbit: injection of 84 mg. myoHb. per kilo body-weight. Death with oliguria and nitrogen retention. No pigment excreted: tendency of urinary urea concentration to recover.
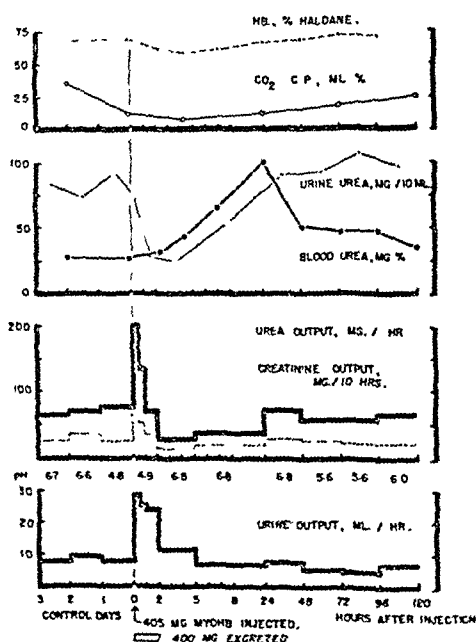
Fig. 4.—"Acid" rabbit: injection of 241 mg. myoHb. per kilo body-weight. Slight impairment of excretion but no oliguria; 84 per cent. of injected pigment excreted in 66 minutes, a further 14 per cent. by 2 hours, none thereafter.

greater than occurred after compression alone (up to 134 mg. per cent.); there was no difference in urinary pH (6·0–6·8 in the first specimen after release) to account for this difference in behaviour, and it was not clearly related to interval after release, duration, or amount of injection. Kidney weights at death were within normal limits. In man the urinary pH following crushing injury falls to very much lower levels (e.g. pH 4·6), and therefore it was thought advisable to follow the results of myoHb. injection without compression in rabbits whose urine had been rendered strongly acid by the ingestion of ammonium chloride.

Group III.   Myohæmoglobin Injection in Rabbits with Urine of Low pH allowed to survive Twenty-four Hours or more.

This group of 27 experiments was subdivided into (a) 13 experiments with unrestricted fluid intake (Group IIIA), (b) 14 experiments with restricted intake (Group IIIB).   With the exception of two animals killed at 24 hours, survival was permitted until the blood urea peak had been attained.   Urinary pH in the first specimen after injection varied between 4·5 and 6·1 (average 5·2).   In 7 experiments no rise in blood urea above 50 mg. per cent. occurred.   In 5 experiments a small rise developed up to 50–100 mg. per cent.   In the remaining 15 experiments the blood urea rose to between 100 and 860 mg. per cent. (average maximum figure being 264 mg. per cent.), and this was accompanied by a depression of renal function, as evidenced by oliguria (fig. 3) and failure to concentrate urea or excrete creatinine.   There were four deaths from uræmia, at 68, 100, 100, and 110 hours after injection, two in each subgroup.   There was a marked tendency for functional renal recovery to ensue, not only when the kidneys were mildly affected (fig. 4), but also when they were more seriously damaged (fig. 5), and even in two of the four experiments where death in uræmia ensued (fig. 6).   In three of the four fatal experiments the maximum blood urea was 300 mg. per cent., and in the other it rose to 860 mg. per cent. In the severely uræmic rabbits there was sometimes a temporary fall in hæmoglobin (about 1–2 g. per cent.), which, in those animals allowed free access to water, returned to the previous level within 48 hours. Plasma protein showed no change (fig. 5).   In those animals which developed no rise in blood urea there was only a small fall in Hb., averaging under 1 g. per cent., similar in magnitude to that produced by control blood sampling.   Serum levels of myoHb. as high as 158 mg. per cent. were produced.   The incidence of high maximum blood urea levels in the two subgroups was not markedly different, but tended to be more frequent in the group on restricted fluid (Table I).   In those rabbits which had shown high blood urea levels the kidneys were found to be markedly swollen and tense, with a slightly mottled surface.   The kidney weights were correspondingly increased (Table IIA) compared
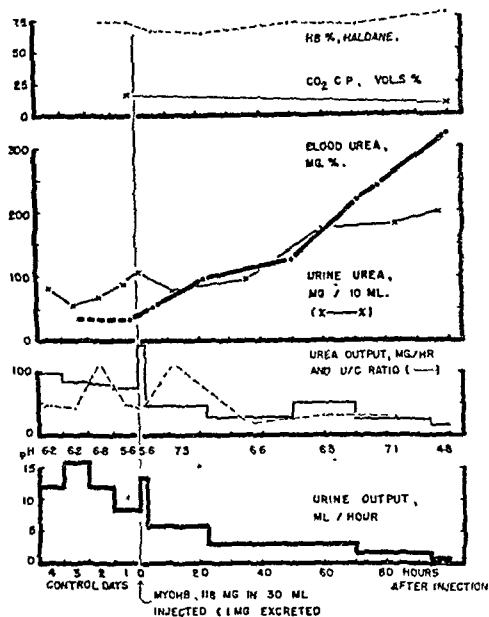
EFFECT OF MYOHB IN THE "ACID" RABBIT.

Fig. 3.—"Acid" rabbit: injection of 84 mg. myoHb. per kilo body-weight. Death with oliguria and nitrogen retention. No pigment excreted: tendency of urinary urea concentration to recover.

Fig. 4.—"Acid" rabbit: injection of 241 mg. myoHb. per kilo body-weight. Slight impairment of excretion but no oliguria; 84 per cent. of injected pigment excreted in 66 minutes, a further 14 per cent. by 2 hours, none thereafter.

Bywaters
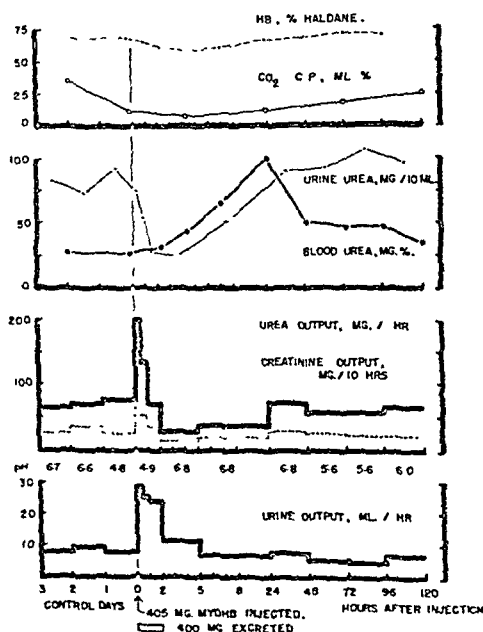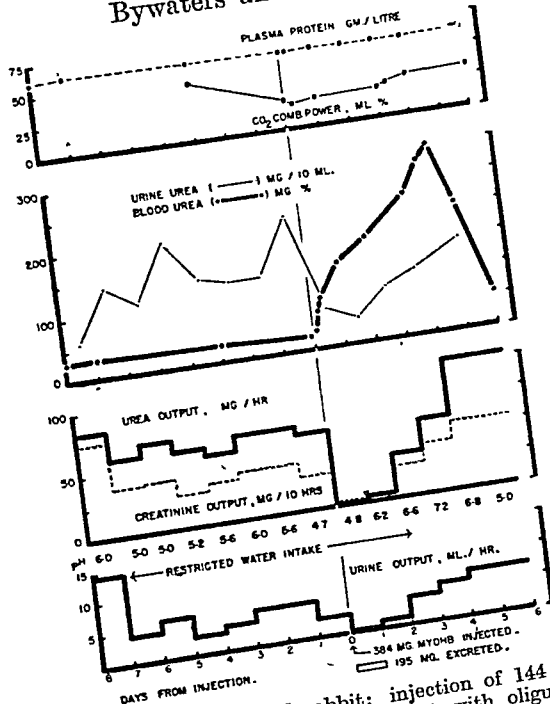
FIG. 5.—"Acid" and dehydrated rabbit: injection of 144 mg. myoHb. per kilo body-weight. Severe excretory impairment with oliguria and recovery. 51 per cent. of injected pigment was excreted by 24 hours, none thereafter.
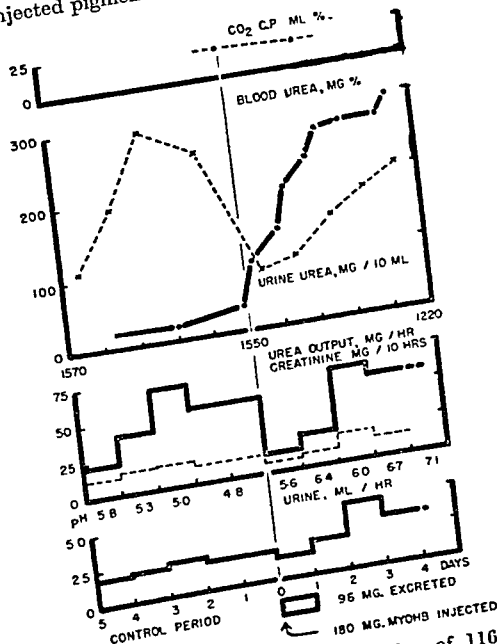
FIG. 6.—"Acid" and dehydrated rabbit: injection of 116 mg. myoHb. per kilo body-weight. 53 per cent. excreted by 22 hours, none thereafter. Death with signs of beginning tubular recovery.

TABLE I.—RELATION OF BLOOD UREA RISE TO FLUID INTAKE (GROUP III).

	Maximum blood urea.	
	Below 100 mg. per cent. No. of experiments.*	100 mg. per cent. or above. No. of experiments.
Acid Diet (Group IIIA).	5 (Mean blood urea: 46 mg. per cent. Mean pigment injected: 120 mg./kg. b.wt.)	6 (Mean blood urea: 199 mg. per cent. Mean pigment injected: 178 mg./kg. b.wt.)
Acid Diet and restricted fluid intake (Group IIIB).	4 (Mean blood urea: 73 mg. per cent. Mean pigment injected: 135 mg./kg. b.wt.)	9 (Mean blood urea: 308 mg. per cent. Mean pigment injected: 112 mg./kg. b.wt.)

χ^2 Test shows no significant difference between Groups IIIA and IIIB in respect to blood urea rise.

* Three experiments with amounts of pigment injected below 50 mg. per cent. are omitted.

TABLE IIA.—RELATION BETWEEN BLOOD UREA RISE AND KIDNEY WEIGHTS (GROUP III).

	Maximum blood urea.	
	Below 100 mg. per cent. No. of experiments.	100 mg. per cent. or above. No. of experiments.*
Kidney weights below 8 g./kg. b.wt.	5	2
Kidney weights above 8 g./kg. b.wt.	1	12
Mean kidney weight: g./kg. b.wt.	6·35	9·5 ± S.D. 1·2

$\chi^2 = 4·0$: Standard error of difference between normal and uræmic mean kidney weights $= 1·5$.

* Omitting an experiment with survival to the 16th day.

with a normal figure of $6·0 \pm$ St. Dev. 0·9 g./kg. body wt. No relation could be found in these 27 experiments between the amount of pigment injected and the maximum blood urea figure (Table I), nor could a significant relation be made out between the amount of pigment excreted and the blood urea rise (Table IIo); of those five who excreted less than 1 mg., three had received very small injections (26–59 mg./kg.),

and the other two died in uræmia. There is, however, a significant relation between the amount of pigment retained and the blood urea rise (Table IIB and fig. 7). The excretion of pigment was completed

FIG. 7.—Retention of pigment and maximum blood urea concentration attained in 35 experiments. One animal (with query) showed a trace of albumen and a blood urea of 92 mg. per cent. in the control period due to over-dehydration. Two of the acid dehydrated series were killed at 24 hours (crosses).

usually between the second and the sixth hours. (There was one exception to this, in which some pigment was excreted after 23 hours.) Thus, changes in the kidney could be investigated within the first 6 hours, using pigment retention as an indication of probable uræmia, had survival been permitted.

TABLE IIB.—RELATION BETWEEN BLOOD UREA RISE AND PIGMENT RETENTION (GROUP III).

	Maximum blood urea.	
	Below 100 mg. per cent. No. of experiments.*	100 mg. per cent. or above. No. of experiments.
Pigment retained below 35 mg./kg. b.wt.	8	2
Pigment retained above 35 mg./kg. b.wt.	1	13
Mean pigment retained.	18 mg./kg. b.wt.	67 mg./kg. b.wt.
$\chi^2 = 10 \cdot 2$		

* Three experiments have been omitted with amounts injected below 50 mg./kg. b.wt.

TABLE IIc.—Relation between Blood Urea Rise, Pigment Excretion, and Urinary pH (Group III).

	Maximum blood urea.	
	Below 100 mg. per cent. No. of experiments.*	100 mg. per cent. or above. No. of experiments.
Pigment excreted below 100 mg./kg. b.wt.	4	11
Pigment excreted above 100 mg./kg. b.wt.	5	4
Mean pigment excreted.	107 mg./kg. b.wt.	71 mg./kg. b.wt.
Mean pigment injected.	127 mg./kg. b.wt.	138 mg./kg. b.wt.
Mean pH of first urine after injection.	5·3	5·1

There is no significant relation shown by the Chi square test between pigment excreted or pH and blood urea rise.

* Three experiments omitted with injected pigment below 50 mg./kg. b.wt.

Group IV.   Myohæmoglobin Injection in Rabbits with Urine of low pH with restricted Water Intake killed within 8 Hours of Injection.

A further series of 12 similar experiments was therefore done to see when the kidney swelling developed: in six of these, killed between 6 and 7 hours of the injection, the kidney was markedly swollen and weighed over 8 g./kg., mean 9 g./kg., the remainder averaging only 6·3 g./kg.   The relationship obtained, if amount of pigment retained

TABLE IID.—Relation between Kidney Weight and Pigment Retention (Groups III and IV).

	Kidneys, g./kilo body-weight.	
	Below 8·0. No. of experiments.*	8·0 and above. No. of experiments.
Pigment retained below 35 mg./kg. b.wt.	10	1
Pigment retained 35 mg./kg. b.wt. or above.	3	18
$\chi^2 = 14·6$		

* Omitting experiment with survival for 16 days.

and the other two died in uræmia. There is, however, a significant relation between the amount of pigment retained and the blood urea rise (Table IIB and fig. 7). The excretion of pigment was completed

FIG. 7.—Retention of pigment and maximum blood urea concentration attained in 35 experiments. One animal (with query) showed a trace of albumen and a blood urea of 92 mg. per cent. in the control period due to over-dehydration. Two of the acid dehydrated series were killed at 24 hours (crosses).

usually between the second and the sixth hours. (There was one exception to this, in which some pigment was excreted after 23 hours.) Thus, changes in the kidney could be investigated within the first 6 hours, using pigment retention as an indication of probable uræmia, had survival been permitted.

TABLE IIB.—RELATION BETWEEN BLOOD UREA RISE AND PIGMENT RETENTION (GROUP III).

	Maximum blood urea.	
	Below 100 mg. per cent. No. of experiments.*	100 mg. per cent. or above. No. of experiments.
Pigment retained below 35 mg./kg. b.wt.	8	2
Pigment retained above 35 mg./kg. b.wt.	1	13
Mean pigment retained.	18 mg./kg. b.wt.	67 mg./kg. b.wt.
$\chi^2 = 10 \cdot 2$		

* Three experiments have been omitted with amounts injected below 50 mg./kg. b.wt.

THE EFFECT OF INCREASED ACTIVITY OF THE SYMPATHETIC SYSTEM IN THE INHIBITION OF WATER-DIURESIS BY EMOTIONAL STRESS. By W. J. O'CONNOR[1] and E. B. VERNEY. From the Pharmacology Laboratory, Cambridge.

RYDIN and VERNEY [1938] produced evidence of an indirect kind that the inhibition of water-diuresis by emotional stress in the dog was due to the release of antidiuretic substance from the posterior lobe of the pituitary, and later work by O'Connor and Verney [1942] established by direct experiment the correctness of this conclusion in the case of animals in which the kidneys had been denervated and the splanchnic nerves divided. In the course of further work on the liberation of antidiuretic substance during emotional stress two additional facts have been observed. First, a large inhibition due to the release of antidiuretic substance has been found to occur in only one-third of tests on normal dogs, but in all tests after denervation of the kidneys with section of the splanchnics and removal of the anterior part of the abdominal sympathetic chains. Secondly, an inhibition of water-diuresis has been observed which has characteristics entirely different from the inhibition due to the release of antidiuretic substance from the posterior lobe of the pituitary. It is the purpose of this paper to present experiments which establish the nature of the new type of inhibition, and also explain the absence of a large inhibition in many tests in normal dogs, as contrasted with its regular appearance after denervation of the kidneys and suprarenals.

METHODS.

These were the same as those described by O'Connor and Verney [1942].

RESULTS.

Inhibition of Water-Diuresis in Normal Dogs.

In fig. 1 are shown four inhibitions of water-diuresis by emotional stress from a series of tests carried out on the same bitch over a period

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[1] Beit Memorial Research Fellow.

be plotted against kidney weight, coincides in these 12 experiments with that found in Group III; the relationship in the two groups combined is analysed in Table IID and depicted, together with that in four normal-diet rabbits in fig. 8.

In a few human cases [Bywaters, 1943] it seemed that the severity of the renal injury ran parallel with the duration of albuminuria.
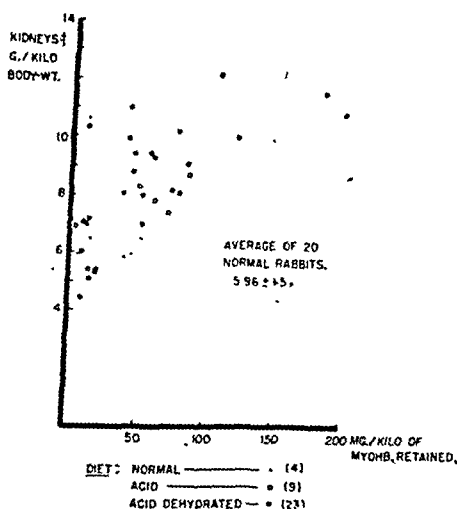
FIG. 8.—Retention of pigment and kidney weight, per kilo body-weight in 4 rabbits on normal diet, 9 on acid diet, and 23 acid-dehydrated animals (one of the last with query, as in fig. 7). Average of 20 normal rabbits is 6·0 g. per kilo body-weight.

TABLE III.—RELATION OF BLOOD UREA RISE TO DURATION OF ALBUMINURIA.

	Maximum blood urea.	
	Below 100 mg. per cent. No. of experiments.	100 mg. per cent. or above. No. of experiments.
Albuminuria for less than 48 hours.	13	1
Albuminuria for more than 48 hours.	4	10
$\chi^2 = 9\cdot6$		

Table III shows that in rabbits also the duration of albuminuria is related to the blood urea rise. The albuminuria lasts longer than pigment excretion (fig. 9).

An analysis of 70 pigmented urines, in which the amounts of soluble

period of stimulation, and that recovery towards the diuretic level is slow, a rate of 2 c.c./min. not being reached again until 40 minutes after the stimulus. Fig. 1, b shows an inhibition of similar maximal intensity but of entirely different time course. In this instance electrical stimulation was applied for 60 sec.: the urine flow fell immediately to the minimum, and during the first $1\frac{1}{2}$ minutes after the end of the stimulation the average rate of flow was only 0·5 c.c./min. A rapid recovery towards the diuretic rate then followed, and 8 minutes after the stimulus (the time of the greatest inhibition in fig. 1, a) the rate was 2·3 c.c./min. The contrast between the time courses of these two inhibitions is clearly shown by reference to the vertical lines drawn in the graphs at 2 and 8 minutes after the end of the stimulus.

Thus are found in the same dog two inhibitions of quite distinct time characteristics—a *rapid* inhibition which reaches its maximum immediately after the stimulus and which has practically vanished within 10 minutes, and a *slow* inhibition which only reaches its greatest intensity in about 8 minutes and from which the rate of urine flow only slowly recovers. In the majority of the tests on the 21 normal bitches used in this investigation, the response was predominantly of one or other of these two types; but in a few instances emotional stress caused an inhibition of water-diuresis in which both types could be clearly seen. One such example, obtained in the same animal as were the responses in fig. 1, a and b, is shown in fig. 1, c. The first stimulus of 30 seconds duration, applied 43 minutes after the test dose of water, resulted in only a small rapid inhibition. A second and stronger stimulus, also lasting 30 seconds, produced first a rapid inhibition in which the flow was only 1 c.c./min. 2 minutes after the end of the stimulation; the rapid recovery from this was, however, interrupted by a slower inhibition in which the rate of flow fell to 1·4 c.c./min. 10–12 minutes after the stimulus. Thereafter the rate of flow steadily recovered towards its diuretic level. There were, then, in this test two phases of inhibition, the first corresponding in its time course with the rapid type of inhibition illustrated in fig. 1, b, the second corresponding with the slow inhibition of fig. 1, a. In all, 12 tests of the effect of faradic stimulation on water-diuresis were made with this animal before operation; 7 of these gave inhibitions of the rapid type with no readily distinguishable slow component; in one test (fig. 1, c) the inhibition was clearly a compound of fast and slow types, the slow component being of small degree; and the remaining 4 tests gave large slow inhibitions in which recovery from any rapid component would be obscured.

Of 21 bitches which have been used, only 3 gave regularly a large slow inhibition of water-diuresis of the type of fig. 1, a in response to 30–60 seconds faradic stimulation; 7 of the bitches always gave a rapid inhibition of the type of fig. 1, b or the first response in fig. 1, c; the

of five weeks, and before any operative interference other than peri-
neotomy. Fig. 1, *a* shows a response similar to that described by
Rydin and Verney [1938]: 43 minutes after the test dose of water the
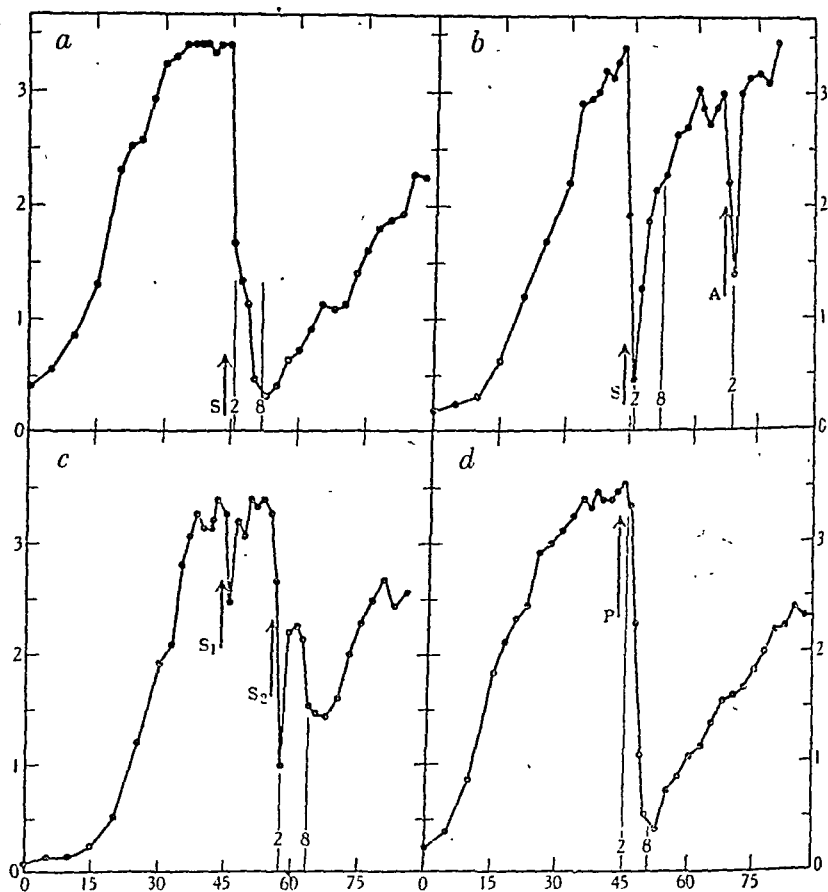
FIG. 1.—Inhibitions of water-diuresis by emotional stress in a normal bitch,
"Slops," wt. = 9·5 kg. (*a*) 7/8/41: at S, 30 seconds faradic stimulation by
needle electrodes inserted through the skin of the flanks. (*b*) 11/9/41: at S,
60 seconds faradic stimulation; at A, intravenous injection of 40 *μ*g. adrenaline.
(*c*) 12/8/41: at S_1, 30 seconds mild faradic stimulation; At S_2, 30 seconds
stronger faradic stimulation. (*d*) 11/8/41: at P, intravenous injection of 1·0 mU.
post-pituitary activity. In this and in all subsequent figures, abscissæ are
minutes after the test dose of 300 c.c. water given at zero time; ordinates are the
rates of urine flow in c.c./min. In all cases the vertical lines 2, 8 are drawn 2 and
8 minutes respectively after the end of the stimulus or injection.

rate of urine flow was steady at 3·4 c.c./min.; a weak faradic stimulus
was then applied for 30 sec. by means of needle electrodes inserted
through the skin of the flanks, and the urine flow fell in consequence
to 0·3 c.c./min. It is especially to be noted that the maximum inhibi-
tion of urine flow is not reached until 8 minutes after the end of the

period of stimulation, and that recovery towards the diuretic level is slow, a rate of 2 c.c./min. not being reached again until 40 minutes after the stimulus. Fig. 1, *b* shows an inhibition of similar maximal intensity but of entirely different time course. In this instance electrical stimulation was applied for 60 sec.: the urine flow fell immediately to the minimum, and during the first $1\frac{1}{2}$ minutes after the end of the stimulation the average rate of flow was only 0·5 c.c./min. A rapid recovery towards the diuretic rate then followed, and 8 minutes after the stimulus (the time of the greatest inhibition in fig. 1, *a*) the rate was 2·3 c.c./min. The contrast between the time courses of these two inhibitions is clearly shown by reference to the vertical lines drawn in the graphs at 2 and 8 minutes after the end of the stimulus.

Thus are found in the same dog two inhibitions of quite distinct time characteristics—a *rapid* inhibition which reaches its maximum immediately after the stimulus and which has practically vanished within 10 minutes, and a *slow* inhibition which only reaches its greatest intensity in about 8 minutes and from which the rate of urine flow only slowly recovers. In the majority of the tests on the 21 normal bitches used in this investigation, the response was predominantly of one or other of these two types; but in a few instances emotional stress caused an inhibition of water-diuresis in which both types could be clearly seen. One such example, obtained in the same animal as were the responses in fig. 1, *a* and *b*, is shown in fig. 1, *c*. The first stimulus of 30 seconds duration, applied 43 minutes after the test dose of water, resulted in only a small rapid inhibition. A second and stronger stimulus, also lasting 30 seconds, produced first a rapid inhibition in which the flow was only 1 c.c./min. 2 minutes after the end of the stimulation; the rapid recovery from this was, however, interrupted by a slower inhibition in which the rate of flow fell to 1·4 c.c./min. 10–12 minutes after the stimulus. Thereafter the rate of flow steadily recovered towards its diuretic level. There were, then, in this test two phases of inhibition, the first corresponding in its time course with the rapid type of inhibition illustrated in fig. 1, *b*, the second corresponding with the slow inhibition of fig. 1, *a*. In all, 12 tests of the effect of faradic stimulation on water-diuresis were made with this animal before operation; 7 of these gave inhibitions of the rapid type with no readily distinguishable slow component; in one test (fig. 1, *c*) the inhibition was clearly a compound of fast and slow types, the slow component being of small degree; and the remaining 4 tests gave large slow inhibitions in which recovery from any rapid component would be obscured.

Of 21 bitches which have been used, only 3 gave regularly a large slow inhibition of water-diuresis of the type of fig. 1, *a* in response to 30–60 seconds faradic stimulation; 7 of the bitches always gave a rapid inhibition of the type of fig. 1, *b* or the first response in fig. 1, *c*; the

remaining 11 animals sometimes gave one type, sometimes the other, and, less commonly, mixed inhibitions in which both types could be recognised. In all, 126 tests have been carried out on the 21 animals, and of these 63 are best classed as rapid inhibitions, 30 were large slow inhibitions, and 33 were of mixed type, although the two successive inhibitions were not always as clearly characterized as in fig. 1, c. By variation in the strength or duration of the electrical stimulus attempts were made to obtain regularly a slow response in those animals which gave inconsistent inhibitions: these were unsuccessful. Thus, with the dog "Slops" (fig. 1), a series of tests with a stimulus of 60 seconds duration revealed inhibitions of all types and in about the same proportions as a series of tests with a stimulus of only 30 seconds duration. With repeated tests some dogs showed signs of alarm as soon as preparations were made for the application of the stimulus, while others did not; but in either case it was not possible by repeated testing to obtain more consistent responses. Indeed, we would emphasize that no conditions of electrical stimulation have been found under which one or other type of inhibition was more liable to occur. Emotional stress from the noise of a "Klaxon" horn also produced inhibition of the rapid, the slow, or the compound type.

Rydin and Verney [1938] compared the slow type of inhibition with the inhibition which results from the intravenous injection of a small dose of pituitary (posterior lobe) extract. Their observation of the similarity in the time courses of these two inhibitions has been repeatedly confirmed in this work, the inhibition due to posterior lobe extract[1] also reaching its maximum intensity 7 to 12 minutes after the injection and slowly disappearing, (cf. fig. 1, a and d). Rydin and Verney also investigated the inhibitions of water-diuresis which resulted from brief mechanical compression of the renal artery and from the intravenous injection of adrenaline. Compression of the renal artery during water-diuresis caused cessation of the urine flow from the one kidney for as long as the compression lasted; rapid recovery in the rate of flow began 2–3 minutes after the release of the compression and was complete within 5 minutes (see fig. 19 of the paper by Rydin and Verney, 1938). Adrenaline (20–40 μg.), injected intravenously into a dog during water-diuresis, also produced a transient inhibition, and this fact has been repeatedly confirmed in the present experiments (see figs. 1, b and 6, b). The rapid inhibition of water-diuresis by emotional stress and the inhibitions due to brief compression of the renal artery or the injection of adrenaline have, therefore, one striking similarity in that the recovery of urine flow is rapid and complete. We are thus led to

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[1] The extract was that marketed by Messrs. Burroughs Wellcome under the name "Infundin" and standardized to contain 10 oxytocic units per c.c. The term milli-unit (mU.) is used to indicate the antidiuretic activity of 10^{-4} c.c. of the extract.

the view that the rapid inhibition is the result of vasoconstriction in the kidney and that the slow inhibition is due to the release of an antidiuretic substance from the posterior lobe of the pituitary. Experiments designed to furnish direct evidence in connexion with these hypotheses will now be described.

The Effect of Removal of the Posterior Lobe of the Pituitary.

With the three animals which regularly gave only a large slow inhibition of water-diuresis in response to 30–60 seconds faradic stimulation it was found that, after removal of the posterior lobe of the pituitary, the same stimulus invariably caused no or only a very small slow inhibition; but a rapid inhibition whose recovery before operation had been concealed by the large slow inhibition now appeared. Fig. 2 shows the results of such experiment with the bitch "Little Black." This animal before operation always gave a large and prolonged slow inhibition of water-diuresis in response to 2 or 3 brief (about 1 second duration) electrical shocks at 10-second intervals, the result of one such test being plotted in fig. 2, a. The posterior lobe of the pituitary was then removed, and fig. 2, b and c show the inhibitions produced by the same stimulus 40 and 42 days after operation. In fig. 2, b the inhibition is of the rapid type with no suggestion of a slow component, while in fig. 2, c the rapid inhibition is followed by a slow inhibition which is, however, very small in comparison with that observed before removal of the posterior lobe (fig. 2, a).

In fig. 3 are shown the results of an experiment on an animal where in most tests before operation the rapid inhibition was not obscured by a slow inhibition. Before removal of the posterior lobe a rapid inhibition alone was observed in 5 out of 7 tests, fig. 3, a being a typical example; in the remaining 2 tests mixed inhibitions like that of fig. 3, b were seen. The posterior lobe was then removed, and one month later the results given in fig. 3, c and d were obtained. The slow inhibition was now absent (fig. 3, c), or of small degree (fig. 3, d), but a rapid inhibition at least as large as those observed before hypophysectomy appeared in all tests.

Removal of the posterior lobe, then, abolishes or greatly diminishes the *slow* type of inhibition of water-diuresis, but leaves the *rapid* type of inhibition apparently unchanged. The next experiments were designed to determine the effect of denervation of the kidneys and suprarenals on the rapid type of inhibition on the one hand and on the slow type on the other.
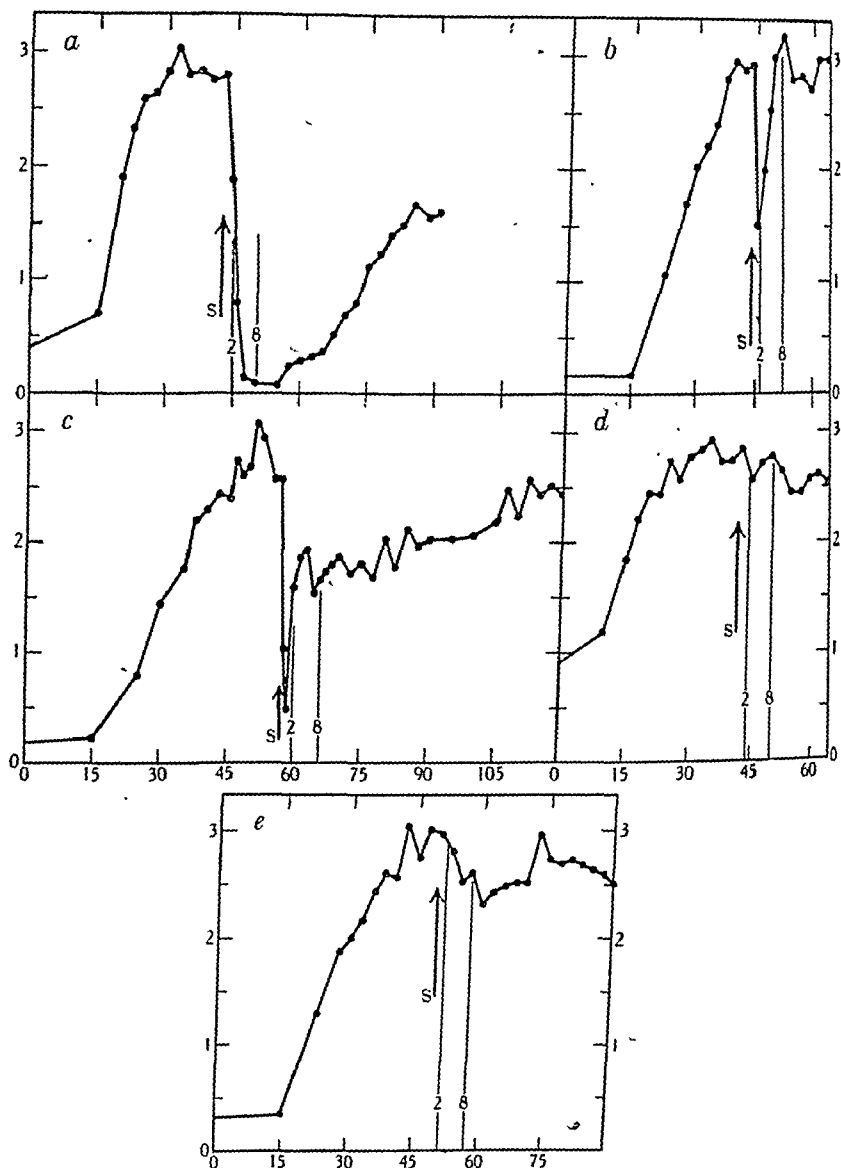
FIG. 2.—Water-diuresis curves illustrating the experiment on the bitch "Little Black," wt. = 10 kg. (a) 15/9/39: before any operation; at S, 2 brief electrical shocks, at 10 seconds interval, each of about 1 second duration. 29/9/39: removal of the posterior lobe of the pituitary. (b) 8/11/39 and (c) 10/11/39: at S, 2 brief electrical shocks. 21/11/39: denervation of kidneys and suprarenals. (d) 1/12/39 and (e) 12/12/39: at S, 2 brief electrical shocks.
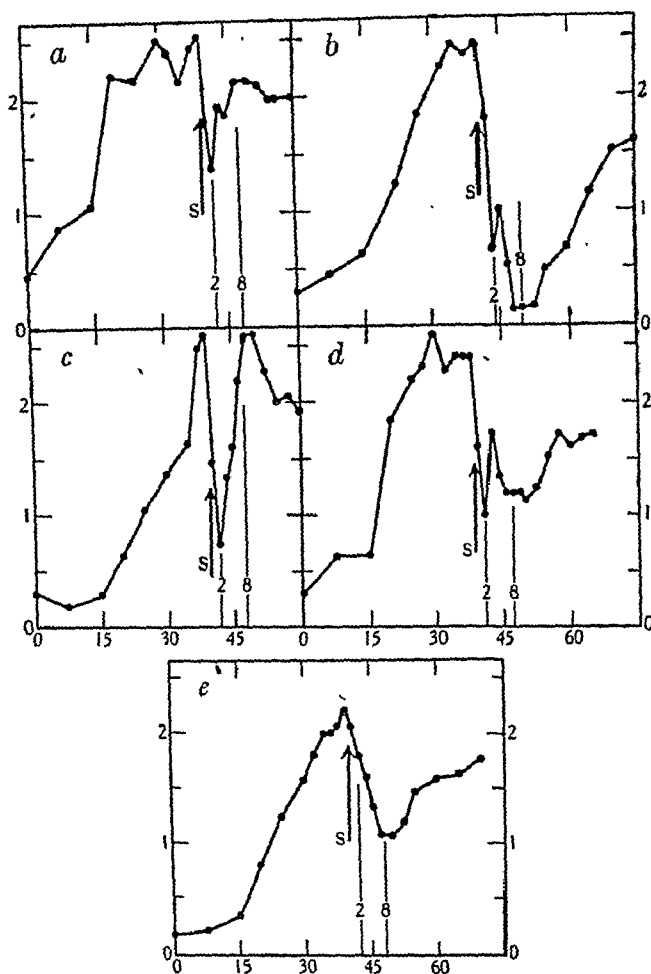
Fig. 3.—Water-diuresis curves illustrating the experiment on "Scutty," wt. = 7 kg. 4/6/43: right kidney removed. (a) 1/10/43 and (b) 30/9/43: at S, 3 brief electrical shocks. 8/10/43: removal of the posterior lobe. (c) 15/11/43 and (d) 9/11/43: at S, 2 electrical shocks. 30/11/43: denervation of the suprarenals and left kidney. (e) 22/12/43: at S, 2 electrical shocks.

The Effect of Section of the Splanchnic Nerves, Removal of the Second, Third, and Fourth Lumbar Sympathetic Ganglia and Denervation of the Kidney.

The operative procedure consisted of: first, the section between ligatures of all connexions of each kidney except the vessels and the ureter, which were then carefully denuded of all visible connective and nervous tissue; secondly, the removal of the second, third, and fourth lumbar sympathetic ganglia on each side together with their interconnexions; thirdly, section of the splanchnic nerves, care being taken

to clear the edge of each crus of the diaphragm and so divide any small twigs passing to either suprarenal gland; and fourthly, replacement of the kidneys into their natural positions by tying together each pair of ligatures between which the peritoneal attachments of the kidneys had been divided. For convenience the operation will be referred to as "denervation," and the indirect evidence which has already been presented would lead one to expect that "denervation" would diminish the rapid inhibition of water-diuresis but would leave the slow inhibition unchanged.

But when "denervation" was performed on dogs with the posterior lobe still intact, an unexpected result appeared. It was found that after "denervation," emotional stress invariably caused a large slow inhibition of water-diuresis, whatever had been the nature of the inhibition before "denervation." In fig. 1 have been given the different types of inhibition produced in "Slops" before any operative interference, a slow inhibition appearing in only 4 out of 12 tests; fig. 4 shows a typical inhibition resulting from the same faradic stimulus after "denervation." All of six tests then resulted in slow inhibitions larger or as large as that shown in fig. 4. The bitch "Tanner II" (fig. 5) provided another example of this effect of "denervation." Fig. 5, a shows a rapid inhibition, which was the only type clearly seen in a series of tests before operation. Fig. 5, b shows the inhibition after "denervation"—a large slow inhibition. The inhibitions in this animal were assayed in terms of post-pituitary extract. Before operation 5 tests were made of the effect of an electrical stimulus of 60 seconds duration, and in each instance any slow component was clearly less than the inhibition produced by the intravenous injection of 0·5 mU. After "denervation" 6 similar tests were made; 5 gave slow inhibitions assayed as the equivalent of 5–10 mU., while in one instance, where the stimulus was a very mild one, the slow inhibition was accurately matched by the inhibition produced by 1·0 mU.

This effect of "denervation" was regularly seen. "Denervation" was performed on 11 dogs, and in every case there was a clear increase in the slow inhibition, so that the stimulus which before operation caused either no slow inhibition or an inconstant one, after operation always caused a large slow inhibition. Later in this paper experiments will be described which elucidate the nature of this new phenomenon.

The large slow inhibition after "denervation" prevents a clear answer to the question whether the rapid inhibition is abolished by the operation designed to prevent vasoconstriction in the kidney during the emotional stress. The question was answered, however, by determining, after removal of the posterior lobe, the effect of "denervation" on the rapid type of inhibition. This was done on the dog "Little Black" (fig. 2). Originally this dog was one of the three in which emotional stress during water-diuresis always resulted in a large slow inhibition;

and attention has already been drawn to the fact that, after removal of the posterior lobe, emotional stress now produced a definite rapid
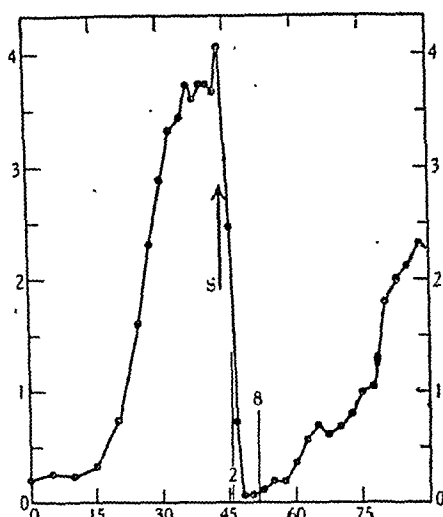
Fig. 4.—Continuation from fig. 1 of the experiment on "Slops." 27/10/41: denervation of the kidneys and suprarenals. 10/11/41: at S, 30 seconds faradic stimulation.
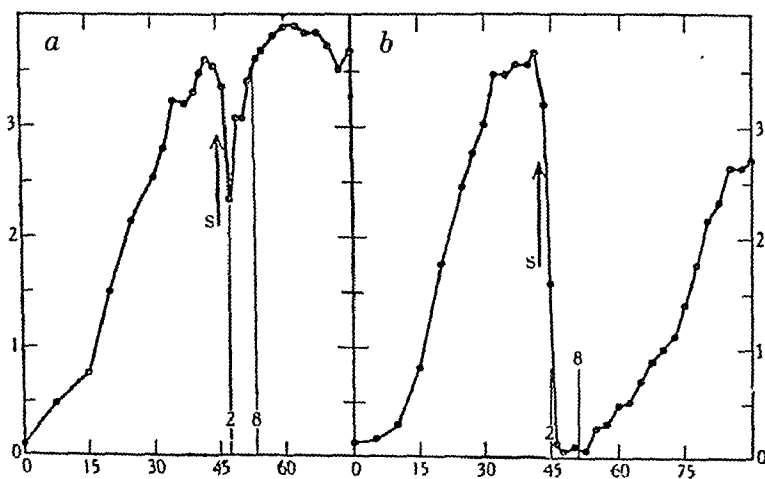
Fig. 5.—Water-diuresis curves from the experiment on "Tanner II," wt. = 10 kg. (a) 21/8/42: before operation: at S, 60 seconds faradic stimulation. 28/10/42: denervation of the kidneys and suprarenals. (b) 24/11/42: at S, 60 seconds faradic stimulation.

inhibition with either no or only a small slow inhibition (fig. 2, b and c). "Denervation" was then performed and the tests were again applied. It was now found that either no inhibition (fig. 2, d) or only a small

slow inhibition occurred (fig. 2, e). Thus in this animal the rapid inhibition was abolished by removal of the nerve supply to the kidneys and suprarenal glands. Another similar experiment was carried out on "Scotty." (fig. 3); in this case also a large rapid inhibition was observed after removal of the posterior lobe but before "denervation" (fig. 3, c and d). Fig. 3, e shows a typical inhibition by emotional stress after "denervation" and, despite the rather large residual slow inhibition, it is clear that the rapid component has almost or entirely vanished.

Since the rapid inhibition has a recovery course similar to that of the inhibition of urine flow produced by mechanical obstruction of the renal artery or by the injection of adrenaline, and since it is not observed after destruction of the nerve supply to the kidneys and to the suprarenals, the rapid inhibition is presumably due to constriction of the renal vessels. It has been established that the slow inhibition is due to the release of antidiuretic substance from the posterior lobe of the pituitary, and it is now of interest to determine the way in which the nerve supply to the kidneys and suprarenals interferes with the expression of the slow component of the inhibition.

The Effect of Adrenaline on the Inhibition of Water-Diuresis by Emotional Stress and by Pituitary (Posterior Lobe) Extract.

It has been shown earlier in this paper that after "denervation" emotional stress during water-diuresis always results in a slow inhibition of urine flow, even with dogs which before operation gave either no or an irregular slow inhibition. One effect of "denervation" must be to limit the output of adrenaline during the emotional stress; and so the first investigation was to determine whether a slow inhibition could be modified by the injection of adrenaline before the application of the stimulus, as in the following experiment. We have already seen that, with the dog "Tanner II" before operation, a 60-second stimulus caused only a rapid inhibition of water-diuresis with no sign of a slow component, but that after "denervation" the same stimulus caused a large slow inhibition (fig. 5, a and b). In B of fig. 6, a is shown another large slow inhibition produced after "denervation." The next day the same stimulus was applied at the same time after the test dose of water; but 30 seconds before the beginning of the 60-second stimulus 15 μg. of adrenaline as the tartrate had been injected intravenously; graph A of the figure shows the result. There was first a large rapid inhibition of urine flow due to the adrenaline, but the rate then recovered rapidly and 8 minutes after the stimulus it had reached 75 per cent. of its diuretic value. In B of fig. 6, b is shown another response to emotional stress preceded by 15 μg. adrenaline, and in this instance the inhibition is compared with that resulting from adrenaline alone (graph A): there is very little difference. Thus, if the stimulus is preceded by the

injection of adrenaline, no large slow inhibition appears.   Graph A of
fig. 6, c shows an inhibition produced by the injection of 15 μg. adrena-
line followed 45 seconds later by 0·5 mU. post-pituitary extract at the
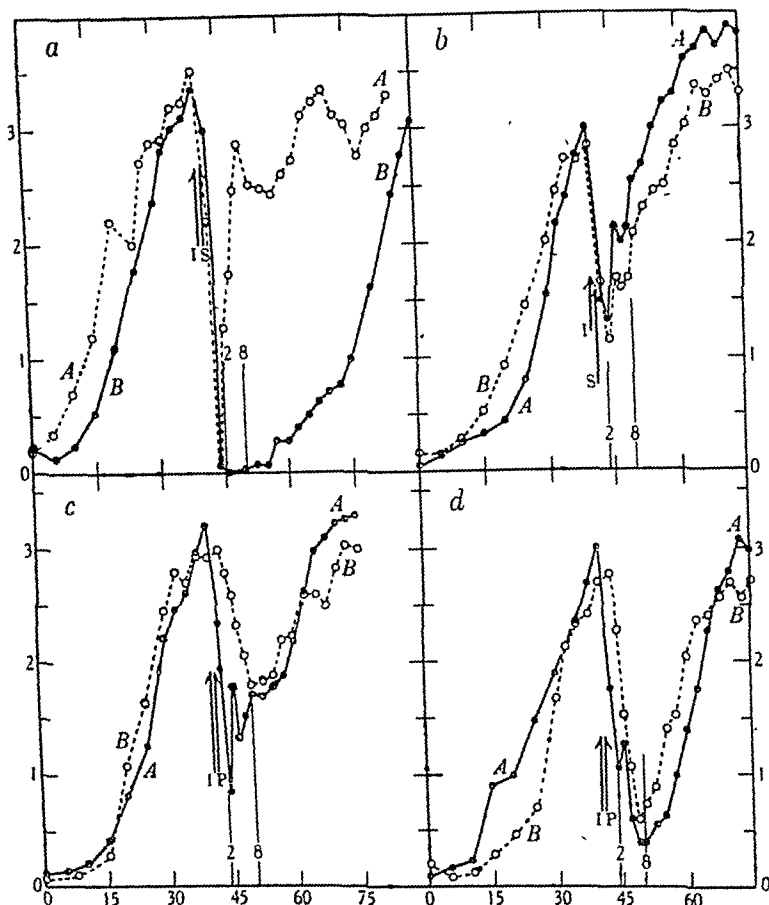
Fig. 6.—Water-diuresis curves illustrating a further experiment on "Tanner II."
28/10/42: denervation of kidneys and suprarenals. (a) Graph A, 26/11/42: at I,
intravenous injection of 15 μg. adrenaline in 0·5 c.c. of 0·9 per cent. NaCl, followed
30 seconds later (S) by 60 seconds faradic stimulation. Graph B, 27/11/42: the
same experiment as on 26/11/42 but at I, 0·5 c.c. of 0·9 per cent. NaCl was
injected. (b) Graph A, 30/11/42: at I, 15 μg. adrenaline intravenously. Graph B,
1/12/42: at I, 15 μg. adrenaline intravenously, followed 30 seconds later at S by
60 seconds faradic stimulation (as in the experiment of 26/11/42). (c) Graph A,
2/12/42: at I, 15 μg. adrenaline, followed 45 seconds later at P by 0·5 mU. post-
pituitary activity. Graph B, 17/12/42: at P, 0·5 mU. alone. (d) Graph A,
3/12/42: at I, 15 μg. adrenaline intravenously, followed 30 seconds later at P
by 1·0 mU. post-pituitary activity. Graph B, 4/12/42: at P, 1·0 mU. alone.

same time after the administration of water as the injections and
stimulations in fig. 6, a and b. Comparison of fig. 6, b and c shows that
the recovery of the inhibition is definitely slower in A, fig. 6, c, than
in B, fig. 6, b, so that any slow component produced by the stimulus

after the injection of adrenaline is less than the slow inhibition produced by 0·5 mU. of the extract. On the other hand, if the stimulus were applied with no injection or after an injection of physiological saline the slow inhibition which resulted was equivalent to that produced by between 5 and 10 mU. Three tests on "Tanner II" (after denervation) of the effect of stimulation preceded by adrenaline all gave inhibitions barely different from the inhibition resulting from adrenaline alone, any slow component being of the same order as the inhibition produced by 0·3 mU. of the extract. This experiment has been attempted on 4 dogs, and in 3 of them the prevention of a slow inhibition by doses of 15 and 20 μg. adrenaline was clearly shown. With the third animal ("Slops"; figs. 1 and 4) no such effect with doses up to 40 μg. was obtained. Figs. 1 and 4 show that after "denervation" the slow inhibition was indeed greatly increased in this animal, and it was expected that this increase would be suppressed or diminished by adrenaline. We are unable to account for the failure to demonstrate such an effect in this dog, unless it be that as a result of the animal's experience of the experimental procedure and consequent foreknowledge of its probable course, the release of pituitary antidiuretic substance was proceeding before the actual injection of the drug.

Thus, after splanchnic section and denervation of the kidneys and suprarenals, when emotional stress during water-diuresis invariably causes a large slow inhibition, adrenaline injected before the stimulus usually prevents the slow inhibition from appearing. Seeking the explanation of this action of adrenaline two possibilities come to the mind: either the adrenaline interferes with the liberation of the antidiuretic substance from the posterior lobe during emotional stress, or the antidiuretic substance is liberated but is unable to act on the kidney in the presence of adrenaline. The results given in fig. 6, c and d lend no support to the second of these possibilities. In fig. 6, c comparison is made between the inhibition produced by 0·5 mU. post-pituitary extract and that produced by the same dose preceded by 15 μg. adrenaline. It is apparent that the inhibitions differ only during the first 8 minutes, while the rapid inhibition due to the adrenaline is still present. Thereafter the inhibitions are of equal degree and follow a similar time course. Again, in fig. 6, d adrenaline has not significantly altered the inhibition produced by 1·0 mU. of post-pituitary activity. This observation has been repeated many times with doses of post-pituitary extract ranging from 0·5–10 mU. and with differing time intervals between the injections, but never has evidence been found that the inhibition of water-diuresis by post-pituitary extract is modified by the injection of 15–40 μg. adrenaline. The failure of the slow inhibition to appear when emotional stress is produced after the injection of adrenaline cannot, then, be explained by an inability of released antidiuretic substance to act on the kidney already under the influence

of adrenaline.   Unless one postulates a difference in this respect between the antidiuretic substance released from the gland in the body and that present in extracts of the posterior lobe, one must conclude that the failure of the slow inhibition to appear in the experiments illustrated in fig. 6, *a* and *b* is due to the injected adrenaline preventing the release of antidiuretic substance during emotional stress.

The irregular appearance of the slow (pituitary) inhibition in normal dogs becomes explicable in terms of these findings, the increase in sympathetic activity during the emotional stress inhibiting in varying degree the liberation of antidiuretic substance from the posterior lobe.

DISCUSSION.

Previous papers by Rydin and Verney [1938] and O'Connor and Verney [1942] on the inhibition of water-diuresis by emotional stress have dealt solely with the slow inhibition, and the fact of the release of an antidiuretic substance from the posterior lobe of the pituitary has been discussed in relation to the theory of the control of urine secretion by a hormone of the neurohypophysis.   The two additional observations reported in this paper have each been shown to be manifestations of increased activity of the sympathetic system.   First, a rapid type of inhibition of water-diuresis has been disclosed, and its dependence upon the integrity of the sympathetic supply to kidneys and suprarenals demonstrated.   Secondly, increased sympathetic activity inhibits the release of antidiuretic substance from the posterior lobe during emotional stress, and after "denervation" this effect is reproducible by the intravenous injection of adrenaline immediately before the stimulus is applied. Indeed, the effect of adrenaline followed by 60 seconds faradic stimulation in the animal after "denervation" resembles very closely the effect of the stimulus alone before denervation (*cf.* fig. 6, *b*, graph B, and fig. 5, *a*).   The experiments do not establish that adrenaline is specifically the cause of the failure of release of antidiuretic substance.   Both the injection of adrenaline and the increased sympathetic activity during emotional stress in the normal dog result in an increase in blood pressure, and it is possible that the consequent stimulation of vasomotor receptors leads to the block in the central nervous system which prevents the release of the antidiuretic substance.   Similarly, "denervation" does not enable us to distinguish between the rôle of the renal sympathetic nerves and that of the release of adrenaline, in the rapid type of inhibition observed in the normal animal;   presumably both functions participate.

Some of the animals were kept for as long as 8 months after section of the splanchnics and denervation of the kidneys and suprarenals. It was observed that the rapid inhibition, abolished by "denervation," reappeared in about 4 months, but was still of reduced size 8 months

after the operation. Similarly, one animal kept with intact posterior lobe for 7 months after "denervation" gave throughout a large slow inhibition of water-diuresis in each test of the effect of emotional stress. It is evident that there is only slow and incomplete sympathetic recovery after this operation.

Summary.

1. Tests of the effect of emotional stress produced by 30–60 seconds faradic stimulation during water-diuresis in normal dogs have revealed two types of inhibition of urine flow: a *rapid* inhibition which is abolished by section of the splanchnics and denervation of the kidneys and suprarenal ("denervation"), and a *slow* inhibition due to release of antidiuretic substance from the posterior lobe of the pituitary.

2. In 21 normal bitches, 3 gave large slow inhibitions in all tests, 7 always gave rapid inhibitions only, and 11 gave a large slow inhibition in some tests. After "denervation" a large slow inhibition was found in all tests in all animals.

3. The slow inhibition thus revealed could be prevented by the injection of adrenaline just before the application of the faradic stimulus.

4. Adrenaline did not diminish the inhibition produced by the injection of post-pituitary extract.

5. It was concluded therefore, that after an injection of adrenaline, the resultant absence of the large slow inhibition produced by emotional srtess in the "denervated" animal was due to failure of release of the antidiuretic substance, and not to failure of the released substance to act on the kidney.

6. The irregular appearance of the slow inhibition in normal dogs is thus explicable in terms of failure of the release of antidiuretic substance from the posterior lobe owing to increased sympathetic activity during emotional stress.

7. Evidence has not yet been sought to determine the relative rôles of adrenaline and the renal nerves in the rapid type of inhibition, nor to decide whether the prevention of release of the antidiuretic hormone is due to adrenaline specifically or to an increase in arterial pressure.

REFERENCES.

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RYDIN, H., and VERNEY, E. B. (1938). *Ibid.* **27**, 343.

NORMAL HÆMATOLOGICAL STANDARDS AT AN ALTITUDE OF 5740 FEET (WITWATERSRAND, SOUTH AFRICA).

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INTRODUCTION.

SINCE 1932 several studies of the erythrocyte and hæmoglobin levels in normal subjects living at this altitude have been published. While most of the results indicate that the red-cell count is appreciably higher than that at sea-level, all the figures for the hæmoglobin appear to be the same or even lower than the values at sea-level. This discrepancy is probably due to the fact that the hæmoglobinometers used were based on the principle of colour matching, and the readings on such instruments are subject to great individual variation. The hæmoglobin results in this study were obtained on a Klett-Summerson photo-electric colorimeter. This instrument measures the intensity of the colour by means of a photo-electric cell and is not influenced by any personal factor.

As this accurate instrument was not available to the previous South African authors, its acquisition provided an opportunity to study hæmoglobin standards anew. Erythrocyte levels were re-investigated and normal standards were determined for the mean corpuscular volume, mean corpuscular hæmoglobin, mean corpuscular hæmoglobin concentration, saturation index, volume index, and diameter of the red cells, which until now have not been recorded for the Witwatersrand.

MATERIAL AND METHOD.

The subjects were European males and females, 30 of each sex, between the ages of eighteen and forty years, who enjoyed normal health and had lived at this altitude for at least one year.

The blood was collected between 8 a.m. and 9 a.m. each day. Three c.cm. of blood were withdrawn from the cubital vein with a dry needle and syringe. Care was taken to avoid any stasis by the removal of the tourniquet as soon as the needle found the vein. The needle was removed from the syringe before the blood was transferred to a tube containing 2·4 mgm. of potassium oxalate and 3·6 mgm. of ammonium oxalate. The tube was corked with a clean dry rubber stopper and

after the operation. Similarly, one animal kept with intact posterior lobe for 7 months after "denervation" gave throughout a large slow inhibition of water-diuresis in each test of the effect of emotional stress. It is evident that there is only slow and incomplete sympathetic recovery after this operation.

Summary.

1. Tests of the effect of emotional stress produced by 30–60 seconds faradic stimulation during water-diuresis in normal dogs have revealed two types of inhibition of urine flow: a *rapid* inhibition which is abolished by section of the splanchnics and denervation of the kidneys and suprarenal ("denervation"), and a *slow* inhibition due to release of antidiuretic substance from the posterior lobe of the pituitary.

2. In 21 normal bitches, 3 gave large slow inhibitions in all tests, 7 always gave rapid inhibitions only, and 11 gave a large slow inhibition in some tests. After "denervation" a large slow inhibition was found . in all tests in all animals.

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6. The irregular appearance of the slow inhibition in normal dogs is thus explicable in terms of failure of the release of antidiuretic substance from the posterior lobe owing to increased sympathetic activity during emotional stress.

7. Evidence has not yet been sought to determine the relative rôles of adrenaline and the renal nerves in the rapid type of inhibition, nor to decide whether the prevention of release of the antidiuretic hormone is due to adrenaline specifically or to an increase in arterial pressure.

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Rydin, H., and Verney, E. B. (1938). *Ibid.* **27**, 343.

Factors were obtained by dividing the ordinate by the abscissa. The average of these factors was then adopted as the correct factor and a line drawn on the graph to correspond to it. The maximum error was found to be ±4·5 per cent.

Hæmoglobin.—8 c.cm. of N/10 HCl were measured with a standardized pipette into each of two test-tubes. 0·02 c.cm. of blood was then transferred to each of these tubes of acid, which were immediately corked, shaken, and allowed to stand for 24 hours at room temperature. Readings were then taken with the Klett-Summerson colorimeter using the green filter. The average reading of the two samples was converted into grams of hæmoglobin by multiplying by the factor.

Erythrocyte Count.—Both a Thoma-Zeiss and a Trenner pipette were used on each case. A watchmaker's lens was used to obtain greater accuracy in the filling. The diluent was Hayem's fluid. The charged pipettes were shaken in a mechanical shaker for 15–30 minutes. Two Thoma-Zeiss hæmocytometers without clips were used. These counting chambers and the cover-glasses were cleaned with acetone and polished with chamois leather before each count. Care was taken that Newton's rings were observed when the cover-glasses were placed in position. As soon as the pipette was removed from the shaker, the fluid was expelled by blowing until the bulb was half empty. The hæmocytometer was filled in the usual manner, care being taken to avoid any air bubbles or overflow into the gutters, and after a few minutes the cells were counted. An area corresponding to 240 small squares or 0·6 sq. mm., *i.e.* approximately 1500 cells, was counted in each chamber. Liknaitzky [1934] showed that if 200 squares are counted, 9 per cent. of determinations will have an error of more than 250,000 per c.mm. and 0·8 per cent. more than 400,000 per c.mm. Ponder [1934] states that if 1000 red cells are counted the error is ±3 per cent., and Osgood [1940] found that if 1200 cells are counted the true count lies within 6 per cent. of the figure obtained. It would appear, therefore, that if 480 squares (or approximately 3000 cells) are counted the error is practically negligible.

The total figure from each counting chamber was divided by 3 and multiplied by 10,000, giving the result in erythrocytes per c.mm. If the result of the Zeiss and Trenner pipettes differed by more than 200,000 per c.mm., then the results were discarded and new dilutions prepared. The two figures were then averaged.

The calibration of the hæmocytometers was checked by doing several parallel counts on Bürker-Turk and Levy-Hauser counting chambers with clips.

All pipettes were calibrated with mercury. They were cleaned with bichromate mixture followed by distilled water between each count. They were thoroughly dried by sucking acetone and then air through them by means of a suction pump.

immediately placed in a mechanical shaker and shaken for 10 minutes. This shaking was continued between each withdrawal of a sample.

Calibration of Colorimeter.

Six samples of blood were used. Their hæmoglobin values were determined by means of Van Slyke's oxygen capacity, Wong's iron
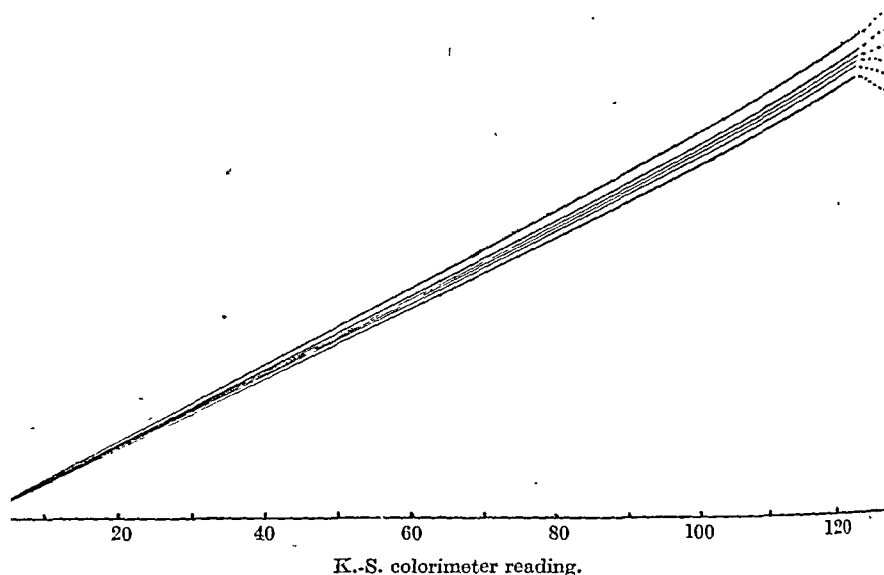
Calibration of Klett-Summerson colorimeter. Acid hæmatin (dilution 1:400). Green filter Factors: Evelyn 0·099, Evelyn 0·102, Van Slyke 0·1025, Pulfrich 0·1037, Wong 0·108. Calculated average factor 0·103.
Maximum error ±4·5 per cent.

a =Evelyn; b =Evelyn; c =Van Slyke; d =average; e =Pulfrich; f =Wong.

estimation, and the alkaline hæmatin methods using the Pulfrich photometer and a standardized Evelyn photo-electric colorimeter. At the same time 0·02 c.cm. of blood from each sample was mixed with 8 c.cm. of N/10 hydrochloric acid. These were corked, well shaken, and allowed to stand at room temperature for 24 hours. After this time, readings were taken on the Klett-Summerson colorimeter using the green filter with a maximum transmission of 540 mμ.

Graphs were then drawn with the hæmoglobin value as the ordinate and the colorimeter reading as the abscissa. Each method of estimating the hæmoglobin was found to give a straight line.

In each case the diameters of 300–400 cells were measured, curves drawn according to Price-Jones, and the mean diameters determined.
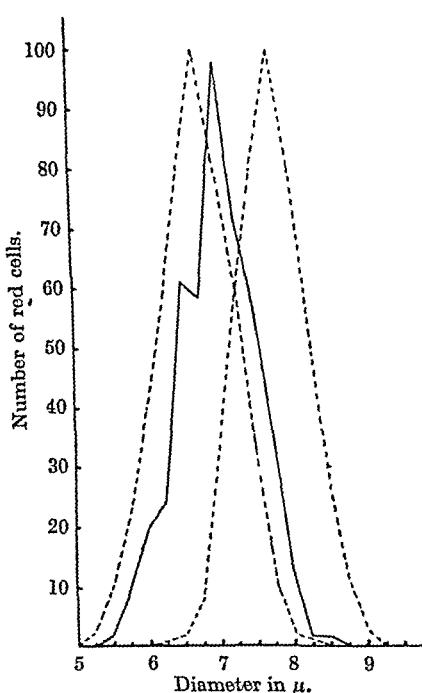
FIG. 4.—Female (subject No. 16).
M = 6·9841 μ.   σ = 0·5298 μ.   v = 7·6 per cent.
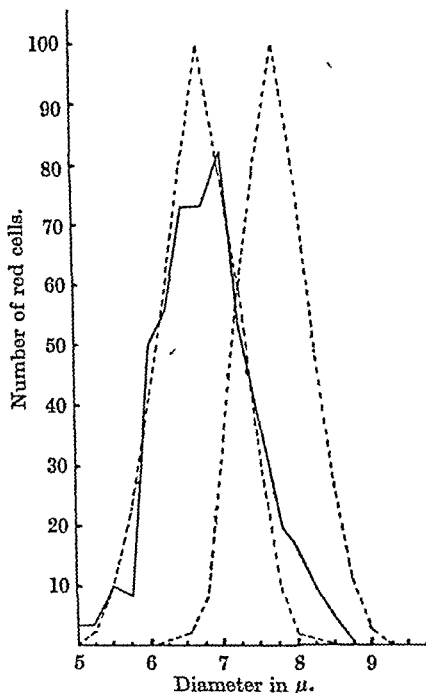
FIG. 5.—Female (subject No. 15).
M = 7·1916 μ.   σ = 0·6473 μ.   v = 9·0 per cent.

Mean Corpuscular Average Thickness (*M.C.A.T.*).—This was calculated on the four cases whose diameters were determined according to the formula:

$$\text{Thickness} = \frac{\text{M.C.V.}}{\pi\left(\dfrac{\text{M.C.D.}}{2}\right)^2}.$$

The results are given in Table I (males), Table II (females), and figs. 2–5 (Price-Jones curves).

Mean Corpuscular Diameter.—The mean diameters as determined by the Price-Jones curves were as follows:—

Males	7·0175 and 6·8366 μ.	Mean 6·92705 μ.
Females	7·1916 and 6·9841 μ.	Mean 7·08785 μ.
	Mean of four 7·0074 μ.	

Packed Cell Volume (P.C.V.).—Wintrobe tubes were used. These were centrifuged at 3000 revolutions per minute for 1½ hours.

The figures in Tables I and II for the *mean corpuscular volume (M.C.V.), mean corpuscular hæmoglobin (M.C.H.), mean corpuscular hæmoglobin concentration (M.C.H.C.), saturation index (S.I.), volume*
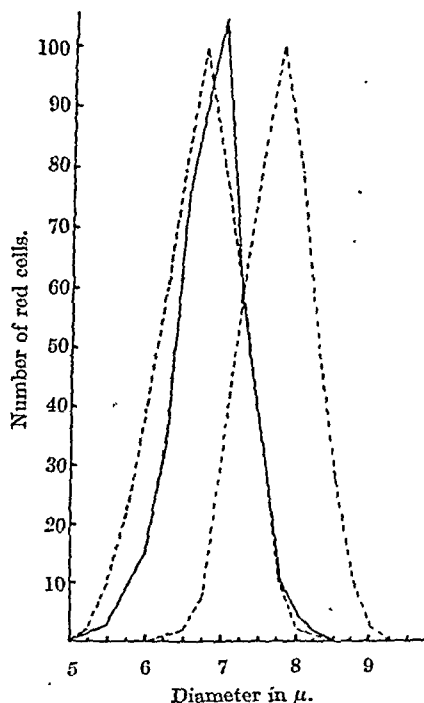
Fig. 2.—Male (subject No. 1).

$M = 6 \cdot 8366 \, \mu.$ $\sigma = 0 \cdot 04658 \, \mu.$ $v = 6 \cdot 8$ per cent.
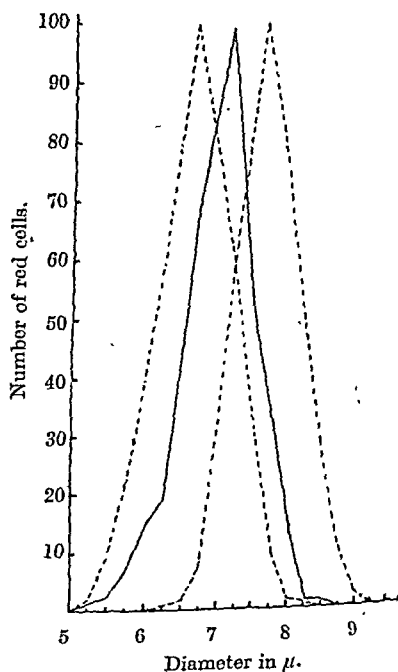
Fig. 3.—Male (subject No. 21).

$M = 7 \cdot 0175 \, \mu.$ $\sigma = 0 \cdot 5222 \, \mu.$ $v = 7 \cdot 4$ per cent.

index (V.I.), and *colour index (C.I.)* were obtained by the usual calculations. In the case of the saturation, volume, and colour indices the normal average figures used were those obtained in this study, not those given by overseas workers.

Mean Corpuscular Diameter (M.C.D.).—Four subjects, 2 males and 2 females, were chosen at random. Thin blood films were made and immediately fixed with Leishman's stain for one minute. They were then washed with buffer solution diluted 1:1 with distilled water. After drying they were stained with 1 per cent. aqueous solution of eosin for one minute and again washed with the same buffer solution. The films were then allowed to dry.

TABLE I.—MALES.

Summary of results derived from 30 healthy adult males between the ages of eighteen and forty years living on the Witwatersrand (altitude 5740 feet).

	Hæmoglobin in g. per cent.	Erythrocytes per c.mm.	Packed cell volume, per cent.	Mean corpuscular volume, c. μ.	Mean corpuscular hæmoglobin, γγ.	Mean corpuscular hæmoglobin concentration, per cent.	Saturation index.	Volume index.	Colour index.
Mean	17·76	5,593,000	50·1	89·58	31·69	35·47	1·0	1·0	1·0
Standard deviation	0·8067	239,600	1·968	2·474	1·018	0·9058	0·02543	0·02708	0·03326
Range as found	15·96–19·62	5,010,000–6,168,000	46·0–55·0	83·0–93·8	29·5–33·6	33·5–37·1	0·95–1·05	0·93–1·05	0·93–1·06
Probable range	15·34–20·18	4,874,000–6,312,000	44·2–56·0	82·16–97·0	28·64–34·74	32·75–38·29	0·92–1·08	0·92–1·08	0·9–1·1
Standard error	0·1472	43,754	0·3593	0·4517	0·1858	0·1653			

Mean Corpuscular Average Thickness.—The results were as follows:—

Males	$2.28\ \mu$ and $2.47\ \mu$.	Mean $2.375\ \mu$.
Females	$2.22\ \mu$ and $2.29\ \mu$.	Mean $2.255\ \mu$.
	Mean of four $2.31\ \mu$.	

In view of the close agreement of three results and the discrepancy of the fourth, an average of the three was also determined with a result of $2.26\ \mu$.

DISCUSSION.

Red-Cell Count.—The significant variations in the red-cell count obtained by the different workers on the Witwatersrand require consideration. The total number of red cells counted by the several investigators may account for variations in the results obtained. Emmerson [1932], Liknaitzky [1934], and Symons [1939] based their results on a count of 500–600 cells, whereas Murray and Lurie [1940] and Gorohovsky [1941] counted 1000, and in this study 3000 red cells were counted in each case. Again, Emmerson, Liknaitzky, Symons, and Gorohovsky used capillary blood, while Murray and Lurie and this study used venous blood. Price-Jones, Vaughan, and Goddard [1935], however, reported no significant difference in counts made from venous and capillary blood. The combination on the one hand of a count of 500–600 cells made with capillary blood, and on the other a count of 1000 and 3000 cells with venous blood may be factors accounting for the significant variations.

It is undisputed that the red-cell count increases with altitude, but the ratio of the rise in the red-cell count to the rise in altitude is not definite. Osgood and Ashworth [1937] state that the red cells increase *by 50,000–100,000 per c.mm. per 1000 feet above sea-level. In Table V* a series of values for sea-level is shown, with their corresponding values for this altitude according to the above principle. It will be seen that the results of Emmerson, Liknaitzky, Buchanan [1935], and Symons are slightly higher than the upper limits, while those of Murray and Lurie and Gorohovsky are slightly lower than the lower limits. The figure of 5·593 millions for males and 4·999 millions for females obtained in this study are almost identical with these lower limits. Andresen and Mugrage [1936], giving a figure of 5·42 millions for males at an altitude of 5280 feet (only 460 feet lower than that of the Witwatersrand), support the findings of Murray and Lurie, Gorohovsky, and this study. Their figure of 4·63 millions for females is appreciably lower than the figures given for sea-level (Table V).

Hæmoglobin.—The great difference between the hæmoglobin values reported by all the previous workers on the Witwatersrand and those obtained in this study may be entirely explained by the types of hæmoglobinometers used. All the previous workers, with the exception

TABLE III.—COMPARISON OF THE FIGURES OBTAINED WITH THOSE OF PREVIOUS WORKERS ON THE WITWATERSRAND.

Author.	Source of blood.	Type of haemoglobinometer.	MALES.						FEMALES.					
			No. of subjects.	Haemoglobin in g. per cent.		Erythrocytes in millions per c.mm.		M.C.H.	No. of subjects.	Haemoglobin in g. per cent.		Erythrocytes in millions per c.mm.		M.C.H.
				Range.	Mean.	Range.	Mean.			Range.	Mean.	Range.	Mean.	
Emmerson	Capillary	Oxygen capacity	118		..	4·69–7·62	6·04							
Stammers	Venous		14	13·94–16·56	14·6									
Liknitzsky	Capillary	Haldane	60	12·14–16·28	14·56	5·12–6·82	5·99	24·3						
Buchanan	Capillary	Neoplan	35	14·21–17·69	16·24	5·29–7·02	6·12	..						
Symons	Capillary	Haldane	46	12·7 –17·25	15·04	5·00–7·02	6·02	25	16	13·05–16·67	14·79	4·87–6·17	5·44	25·9
Symons	Capillary	Haldane	46	12·14–17·25	14·75	4·69–7·62	6·02	24·5	16	11·73–15·87	14·08	4·92–6·17	5·44	25·9
Murray and Luria.	Venous	Neoplan	70	11·89–17·57	14·73	4·18–6·8	5·47	26·9	43	9·41–16·53	12·97	4·07–5·92	4·992	
Gorohovsky	Capillary	Hellige Neoplan	100	11·585–17·057	14·62	4·129–6·853	5·491	26·6	50	10·647–15·325	12·986	3·494–6·502	4·998	25·9
This study	Venous	Klett-Summerson	30	15·34–20·18	17·76	4·87–6·31	5·593	31·7	30	12·87–17·79	15·33	4·28–5·71	4·990	30·7

The above figures have been computed to the same bases and do not necessarily correspond to the form in which they were published.

TABLE II.—FEMALES.

Summary of results derived from 30 healthy adult females between the ages of eighteen and forty years living on the Witwatersrand (altitude 5740 feet).

	Hæmo-globin in g. per cent.	Erythrocytes per c.mm.	Packed cell volume, per cent.	Mean corpuscular volume, c.µ.	Mean corpuscular hæmoglobin, γγ.	Mean corpuscular hæmoglobin concentration, per cent.	Saturation index.	Volume index.	Colour index.
Mean .	15·33	4,999,000	45·2	90·42	30·65	33·82	1·0	1·0	1·0
Standard deviation	0·8193	237,000	2·002	1·983	1·109	0·9648	0·02751	0·02306	0·03824
Range as found .	13·91–17·72	4,540,000–5,521,000	41·5–49·0	85·3–93·3	28·1–32·5	31·7–36·2	0·94–1·07	0·94–1·03	0·92–1·06
Probable range .	12·87–17·79	4,285,000–5,713,000	39·2–51·2	84·47–96·37	27·33–33·98	30·93–36·71	0·92–1·08	0·93–1·07	0·89–1·11
Standard error .	0·1496	43,440	0·3655	0·3622	0·2025	0·1762			

TABLE V.—COMPARISON OF THE RESULTS WITH VALUES GIVEN FOR SEA-LEVEL AND THEIR CORRECTION FOR THE ALTITUDE OF THE WITWATERSRAND.

Author.	MALE.								FEMALE.							
	Hæmoglobin in g. per cent. sea-level.	Corrected hæmoglobin in g. per cent.*	Red cells in millions per c.mm. sea-level.	Corrected red cells in millions per c.mm.†	P.C.V. per cent, sea-level.	M.C.V., c.µ.	M.C.H., γγ.	M.C.H.C. per cent.	Hæmoglobin in g. per cent. sea-level.	Corrected hæmoglobin in g. per cent.*	Red cells in millions per c.mm. sea-level.	Corrected red cells in millions per c.mm.†	P.C.V. per cent, sea-level.	M.C.V., c.µ.	M.C.H., γγ.	M.C.H.C. per cent.
Osgood	15·8	18·04	5·4	5·68–5·97	45·0	82·0	29·4	35·1	13·8	15·75	4·8	5·08–5·37	41·0	86·0	28·6	33·7
Osgood, Haskins, and Trotmnn.	15·8	18·04	5·4	5·68–5·97	46·35	85·8	29·2	34·0	13·7	15·64	4·8	5·08–5·37	42·43	88·3	28·5	32·2
Wintrobe	16·0	18·26	5·48	5·76–6·05	47·0	85·7	29·1	34·0	14·1	16·1	4·82	5·1 –5·39	42·0	87·1	29·2	33·5
Wintrobe and Miller	17·0	19·4	5·85	6·13–6·42	49·6	84·7	29·0	34·2	13·76	15·72	4·93	5·21–5·5	41·5	84·1	28·0	33·1
Haden	15·34	17·51	4·95	5·23–5·52	45·5	92·0	31·0	33·7	13·37	15·26	4·38	4·66–4·95	39·8	91·0	30·5	33·5
Price - Jones, Vaughan and Goddard.	14·55	16·61	5·42	5·50–5·99	..	85·92	26·86	31·26								
McGeorge	15·18	17·33	5·48	5·76–6·05	48·2	84·6	27·8	33·0								
Walters	15·12	17·26	4·84	5·12–5·41	46·5	96·5	31·4	32·4								
Foster and Johnson	15·74	17·97	5·26	5·54–5·83	46·7	88·7	29·9	33·6								
Mean.	15·61	17·82	5·34	5·60–5·91	46·85	87·32	29·29	33·47	13·74	15·69	4·74	5·02–5·31	41·34	87·3	28·96	33·2
This study.	..	17·76	..	5·593	..	89·58	31·60	35·47	..	15·33	..	4·999	..	90·42	30·65	33·82

* Fitzgerald's law: For every 100-mm. fall in atmospheric pressure there is an average rise of about 10 per cent. in hæmoglobin.

† Osgood and Ashworth: The red-cell count increases by 50,000 to 100,000 per thousand feet above sea-level.

TABLE IV.—COMPARISON OF THE FIGURES OBTAINED WITH THOSE OF OTHER WORKERS AT APPROXIMATELY SIMILAR ALTITUDES.

Author.	Altitude, feet above sea-level.	MALES.						FEMALES.					
		Haemoglobin in g. per cent.	Red cells in millions per c.mm.	Packed cell volume, per cent.	Mean corpuscular volume, c.µ.	Mean corpuscular haemoglobin, γγ.	Mean corpuscular haemoglobin concentration, per cent.	Haemoglobin in g. per cent.	Red cells in millions per c.mm.	Packed cell volume, per cent.	Mean corpuscular volume, c.µ.	Mean corpuscular haemoglobin, γγ.	Mean corpuscular haemoglobin concentration, per cent.
Hingston	4390	..	5·24										
Starling	5117	..	6·551										
Lewis, Ilift,* Duval, and Kinsman.	5280	16·53	6·07	48·83	80–81	26–28	33–35	13·86	4·84	41·8	81–91	26–30	32–34
Andresen and Mugrage.	5280	16·64	5·42	48·35	89·2 80–100	30·5 27–34	34·2 31–37	14·43	4·63	43·32	93·3 79–100	31·2 27–34	33·4 30–36
This study	5740	17·76	5·593	50·1	89·6 82–97	31·7 28·6–34·7	35·5 32·7–38·2	15·33	4·999	45·2	90·4 84·5–96·4	30·7 27·3–34·0	33·8 30·9–36·7
Starling	5000	..	7·0										
Hingston	8000	..	6·04										

* Two males and five females had been resident at this altitude for only a few weeks.

is for this reason that Liknaitzky, Symons, Murray and Lurie, and Gorohovsky obtained values lower than the lower limit of normal given by other workers (see Table III). Whitby and Britton [1942] give the normal range at 27–32 micromicrograms. While the figures obtained in this study are higher than the mean value for sea-level (see Table V), they are within the normal range. Andresen and Mugrage working at an altitude of 5280 feet give figures also higher than the mean and not very different from those obtained in the present work. It would appear, therefore, that the hæmoglobin value rises proportionately with the red-cell count, or even to a slightly greater extent, so that each red cell at this altitude contains as much as or slightly more hæmoglobin than a red cell at sea-level.

Mean Corpuscular Hæmoglobin Concentration (M.C.H.C.).—The figures obtained in this study are similar to those of Andresen and Mugrage. They are within the normal limits given by Whitby and Britton (32–38 per cent.) and only very slightly higher than the mean (34 per cent.).

Mean Corpuscular Volume (M.C.V.).—Hurtado found that at high altitudes the red cell is larger than at sea-level but contains less hæmoglobin. Smith, Belt, Arnold, and Carrier, on the other hand, found that the red cell gets smaller with rise in altitude but contains as much as or more hæmoglobin than at sea-level. The figures for the M.C.V. obtained in this study are within the normal limits given by Whitby and Britton (78–94 c.μ) but slightly higher than the mean at sea-level (87·3 c.μ). Andresen and Mugrage found the same at Denver.

Mean Corpuscular Diameter (M.C.D.).—Although the volume of each red cell at this altitude is slightly greater than that at sea-level, its diameter is slightly smaller (7·0074 μ). Price-Jones [1933] has shown that the normal range is from 6·7 to 7·7 μ, with an average of 7·2 μ.

Mean Corpuscular Average Thickness (M.C.A.T.).—As the red cell has a slightly larger volume and smaller diameter than that of a red cell at sea-level, its thickness is slightly larger, 2·26 μ. Price-Jones, Vaughan, and Goddard [1935] give the normal range as 1·7–2·5 μ, with a mean of 2·1 μ.

CONCLUSION.

The results of this study indicate that at an altitude of 5740 feet the number of red cells and the hæmoglobin content of the blood is higher than at sea-level. The hæmoglobin rises proportionately with the red-cell count. Each red cell contains as much as or slightly more hæmoglobin than does a red cell at sea-level. The red-cell volume is slightly larger, although its diameter is a little smaller. The thickness is greater than that of an average red cell at sea-level. These findings therefore suggest a tendency to a diminution in the diameter : thickness ratio. At high levels compensation occurs not only by an increase in the number

of Stammers [1933], used either the Haldane or the Neoplan hæmoglobinometer. This study was done with the Klett-Summerson photoelectric colorimeter.

While it is possible that the amount of hæmoglobin does not rise in proportion to the number of red cells with increase in altitude, it is not disputed that there is a rise.

Schneider [1921] states that "the relation between the increase in the number of red-blood corpuscles and amount of hæmoglobin has been a subject of debate. Schaumann and Rosenqvist, Oliver, Van Voornveld, and Fuchs, found that the increase in red cells exceeded that of hæmoglobin, while Eggers and Dallwig, Kolls and Lowenhart, report a much smaller increase in red-blood corpuscles than in hæmoglobin. The Anglo-American Pike's Peak expedition in 1911 found the red-blood corpuscles to increase in equal proportion with the hæmoglobin, so that there was no alteration of the colour index. Bürker and collaborators at 6150 feet found the red-blood corpuscles to increase 4 to 11·5 per cent. and the hæmoglobin 7 to 10 per cent. Cohnheim and Schneider and Havens find the two changes run parallel."

Smith, Belt, Arnold, and Carrier [1925] found that with rise in altitude the increase in the erythrocyte count and in hæmoglobin are almost equal, or if anything the rise in hæmoglobin is slightly greater.

Osgood states that at high altitudes the hæmoglobin is not elevated to as great an extent as the erythrocytes, so that the colour and saturation indices tend to be lower than in persons at sea-level. Hurtado [1932] also found that with rise in altitude the hæmoglobin does not increase proportionately with the red-blood corpuscles.

There is no doubt that the red-cell count on the Witwatersrand is higher than that at sea-level. The figures for hæmoglobin given by Stammers, Liknaitzky, Symons, Murray and Lurie, and Gorohovsky are lower than all except one figure for sea-level in Table V. It would appear, therefore, that their results are too low.

In Table V, Fitzgerald's law, stating that the hæmoglobin rises by 10 per cent. for every 100 mm. mercury fall in barometric pressure, was applied to the sea-level figures. The means of 17·82 g. for males and 15·69 g. for females are almost identical with the results obtained in this study (17·76 g. for males and 15·33 g. for females). Andresen and Mugrage found values of 16·64 g. for males and 14·43 g. for females at an altitude of 5280 feet. These figures also, therefore, appear to confirm the results obtained in the present work. It is worthy of mention that Symons obtained a figure of 16·93 g. of hæmoglobin for males using a colorimeter with a Newcomer standard. This he considered inaccurate, as it was so much higher than his results with the Haldane hæmoglobinometer and his modification of Wong's iron estimation.

Mean Corpuscular Hæmoglobin (M.C.H.).—The values for this are necessarily dependent upon the values for red cells and hæmoglobin. It

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of erythrocytes, but also by an increase in the volume and hæmoglobin content of each cell.

SUMMARY.

1. A hæmatological study is described, based on the examination of normal European males and females, 30 of each sex, between the ages of eighteen and forty years, living at an altitude of 5740 feet.

2. Normal values have been determined for the hæmoglobin, red-cell count, packed cell volume, mean corpuscular volume, mean corpuscular hæmoglobin, mean corpuscular hæmoglobin concentration, mean corpuscular diameter, and mean corpuscular average thickness.

3. The mean red-cell count for males has been found to be 5·593 millions per c.mm. and 4·999 millions per c.mm. for females, with hæmoglobin values of 17·76 g. per cent. and 15·33 g. per cent. respectively. The male red cell has a mean volume of 89·58 c.μ and a mean corpuscular hæmoglobin of 31·69 $\gamma\gamma$. The female red cell has a mean volume of 90·42 c.μ and a mean corpuscular hæmoglobin of 30·65 $\gamma\gamma$.

The mean diameter has been found to be 7·0074 μ and the mean thickness 2·26 μ.

4. These results have been compared with those of previous workers on the Witwatersrand, and other records found at similar altitudes in other parts of the world.

5. The normal values obtained for this altitude have been compared with the normal values at sea-level.

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OBSERVATIONS RELATING TO CARDIAC HYPERTROPHY PRODUCED IN THE RABBIT BY ARTERIO-VENOUS ANASTOMOSIS: THE EFFECT OF CLOSURE OF THE ANASTOMOSIS. By ALAN N. DRURY. From the Department of Pathology, University of Cambridge, and Lister Institute, London.

HOLMAN [1937] came to the conclusion that, when, as a result of an arterio-venous fistula, large hearts are seen clinically and roentgenographically, it is safe to assume that the enlargement is largely due to dilatation and only in moderate degree to hypertrophy. If such is the case, removal of the fistula should restore the heart to normal size. On the other hand, he obtained in dogs, roentgenographic evidence of some slight permanent enlargement in some animals, and the weights of hearts removed at autopsy showed a definite increase in relation to body-weight to that found in normal animals. There is some difficulty in accepting these findings as evidence owing to the fact that the heart-weight to body-weight ratios vary considerably in dogs of different breeds. The work of Hermann [1926] on dogs showed that a relatively high grade of hypertrophy could be produced by carotid jugular fistulæ, and this finding was borne out by the experiments carried out by Drury and Wightman [1940] on rabbits. In that paper the effect of an arterio-venous anastomosis upon the heart was described in some detail. The chief findings reported were that within less than three months of establishing the anastomosis between the carotid artery and jugular vein, the heart may weigh twice the normal weight. This hypertrophy involves all the heart chambers, but while the increase in weight of the auricles may amount to 300 per cent., that of the ventricles seldom exceeds 100 per cent.

In the present paper the effect of tying off the anastomosis upon a heart which is known to be hypertrophied has been investigated. These experiments have been carried out to determine whether hearts which had hypertrophied to a degree reported in the previous paper return to their normal weight, and how long it takes for this to occur.

METHOD.

The anastomosis was established between the right carotid artery and right jugular vein of rabbits in the manner already described [Drury and Wightman, 1940]. Two groups of animals were operated

TABLE I.—AREA OF CARDIAC SHADOW IN THE SAME ANIMALS
X-RAYED ON SUCCESSIVE DAYS.

Animal.	Area of cardiac shadow in sq. cms.
1	9·9, 8·8, 9·1, 8·1, 9·3
2	5·2, 6·6, 6·7, 5·7
3	5·6, 6·4, 5·8
4	9·6, 9·8, 8·3, 9·0
5	8·8, 9·1, 8·1, 9·3
6	7·8, 8·9, 8·4, 8·4

In fig. 1 the actual heart-weights of the control or first series (in which in addition five normal rabbits are included) have been plotted
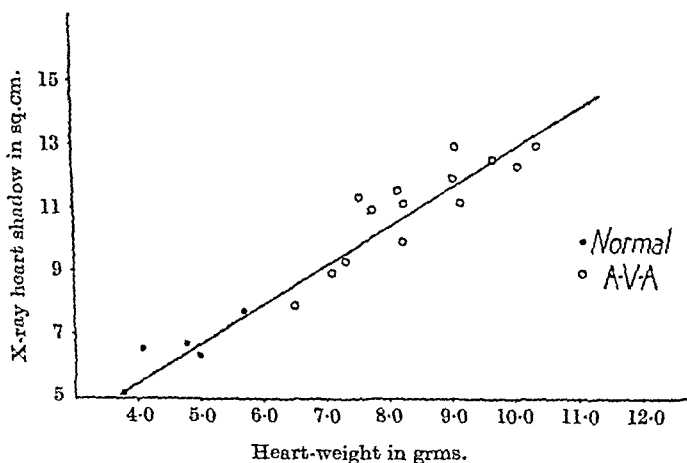
FIG. 1.—Relationship between area of cardiac shadow and actual heart-weight.

against the shadow area. While there is a general relationship between the weight and the area, the spread is such that there is a possible error of at least 1 g. and this, coupled with the fact that the shadow may have a variation of 1 sq. cm. in the same animal, means that the calculated weight may have an error of anything up to 2 g., which in a heart weighing 8–10 g. is considerable. The importance of taking such X-rays lies in the fact that in general the hearts with the largest shadows weigh the most, and in choosing animals in which it is proposed to tie off the anastomosis, it is the most reliable guide that they have hypertrophied hearts. The intensity of the signs such as murmur, thrill, or the cardiac effect produced by closure of the anastomosis, are no indications of the size of the heart, for they can be very intense in an animal in which the leak is relatively small, and with a heart showing little hypertrophy.

upon. In the first group, after the anastomosis had been patent for three months, X-ray photographs of the hearts were taken and the animals were killed on the following day, and the hearts fixed and weighed [Drury and Wightman, 1940]. In this manner it was possible to relate the cardiac area to the actual heart-weight. In the second series, after the anastomosis had been patent for three months, X-ray photographs of the heart were taken, and certain animals were selected in which the cardiac area indicated that hypertrophy was present. These selected animals were operated upon on the day following the taking of the X-ray photograph and the anastomosis closed by ligating the carotid artery. Two animals of this series were killed at varying intervals after the operation and the hearts were fixed and weighed.

Assessment of Heart-Weight from the X-ray Heart Shadow.

When an anastomosis is established, the heart dilates as well as hypertrophies, and unless these two effects move hand in hand, the size of the X-ray shadow cannot be expected to give an accurate assessment of the heart-weight. From observation of the heart *in situ* in the anæsthetised animal, the largest hearts do not necessarily prove to be the heaviest. There are, moreover, technical difficulties in taking X-ray photographs which also introduce errors. It is essential to hold the animal in the vertical position so that the diaphragm falls clear of the heart's apex, and in this position the heart becomes smaller as the blood collects in the dependent parts. It is not possible to ensure that the animals are held in the same position relative to the X-ray tube. To lessen these errors as far as possible, the following standard procedure was adopted. The animal was tied down firmly on a horizontal board with two adjustable side boards which could be moved up to the chest wall on each side, to prevent rotation. The X-ray film was then slipped between the animal's back and the board. The board was then moved to the vertical position and placed at a constant distance of 22 inches from the X-ray tube, which was centred on the mid-sternal point. At the end of ten seconds, the exposure (1/25th of a second) was made, no attempt being made to take the photograph during systole or diastole, as the change in size due to contraction is very small. Outline drawings of the heart shadow were then traced upon tracing paper. Two definite nicks are seen where the right and left auricles join the large vessels, and the outline was commenced at one of these and carried around the whole shadow till the other nick was reached; the nicks being joined by a straight line. The outline was then cut out and weighed, the area being calculated from the weight of a known area of identical paper.

A number of animals were photographed on several successive days and it was found that the difference in the heart-shadow area amounted to about 1 sq. cm. (Table I) in the same animal.

TABLE II.

Rabbit.	X-ray shadow, sq. cm.	Before tying off anastomosis. Estimated values.			After tying off anastomosis. Actual values.				Time animal killed after tying off A.V.A.
		Heart-weight, fig. 1.	$\dfrac{\text{Heart-weight} \times 100}{\text{Carcase-weight}}$ *	Heart-weight increase above normal of Drury and Wightman, 1940. Per cent.	$\dfrac{\text{Heart-weight} \times 100}{\text{Carcase-weight}}$	$\dfrac{\text{Vent. weight} \times 100}{\text{Carcase-weight}}$	$\dfrac{\text{Aur. weight} \times 100}{\text{Carcase-weight}}$	$\dfrac{\text{Left heart weight}}{\text{Right heart weight}}$	
Controls	..	..	..	..	·22–·34	·18–·32	·20–·45	1·9–2·7	..
1	15	11·5	·66	125	·33	·28	·45	2·0	16 weeks
1a	10	7·5	·37	25	·29	·25	·35	2·7	16 ,,
2	13	10·0	·58	100	*·41*	·35	·52	2·0	12 ,,
2a	13	10·0	·58	100	*·34*	·31	·36	1·9	12 ,,
3	11	8·3	·50	73	·31	·23	·31	2·5	10 ,,
3a	12	9·0	·54	86	·34	·30	·34	2·3	10 ,,
4	16	12·0	·70	140	·33	·29	·39	2·0	8
4a	11	8·3	·50	73	*·32*	·28	·34	2·1	8
5	13	10·0	·58	100	·37	·34	·31	2·6	6
5a	11	8·3	·50	73	*·35*	·32	·38	2·1	6
6	13	10·0	·58	100	*·42*	·38	·45	2·3	4
6a	13	10·0	·58	100	*·35*	·33	·33	2·4	4
7	16	12·0	·70	140	*·57*	*·46*	*·97*	2·1	1 week
7a	11	8·3	·50	73	*·43*	*·47*	*·59*	2·4	1 ,,

Values above normal in *italics*.

* The "carcase-weight" of the living animal is arrived at by subtracting from the "live weight" the average weight of the intestines removed from animals of similar weight.

Closure of the Anastomosis.

Animals were specially chosen in which the cardiac area by X-ray was 10 sq. cm. or over. They were X-rayed the day previous to the operation in which the carotid artery was doubly ligated under nembutal anæsthesia. The animals so operated upon were killed in pairs 1, 4, 6, 8, 10, 12, 14 weeks later, and the hearts fixed and weighed. The results are tabulated in Table II. In the second and third columns the estimated *heart-weight* and *heart-weight × 100* to carcase-weight [1] ratios, deduced from the cardiac area before operation, are given. The ratios indicate that in many cases hearts weighing twice the normal value were present [see Drury and Wightman, 1940]. The actual values obtained from the weights of the hearts at different time periods after operation show that with one exception they are all within the normal range after a period of 6–8 weeks had elapsed. In addition, the ratio of left to right heart-weights are also within the normal range, so that all chambers of the heart have been involved in the change. On the other hand, it must be pointed out that although the values are within normal limits, they tend to lie at the top end of the normal scale, and the same is true of all the other ratios given. It would appear, therefore, that the only safe conclusion to draw is that there is a gradual return to normality, but that even at 16 weeks a full return may not have taken place, even though it can be admitted that the values could be normal. It is unlikely that all the ratios would lie at the top end of the normal scale if the hearts had returned their own normality. One animal, No. 2, 12 weeks after the closure gave heart ratios which were well outside the normal limits, indicating that a considerable degree of hypertrophy was still present.

DISCUSSION.

Hypertrophy may be considered physiological if, on removing the stimulus producing it, the organ returns to its normal size. The observations here reported indicate that the hypertrophy produced by a carotid jugular anastomosis is for the most part, if not wholly, physiological in type even if it is such that the weight of the heart is doubled. It is not possible to state categorically that it is physiological in type, for even after 16 weeks a small residuum of hypertrophy may still remain. Whether this would have disappeared if a larger period had been allowed after operation it is not possible to say from these experiments. The time taken for the hypertrophy to disappear is 6–8 weeks, which is very similar to that which it takes to develop. One animal, No. 2, even after 12 weeks still had a considerable degree of hypertrophy. The hypertrophy in this animal may only be pathological in the rate

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[1] The "carcase weight" of the living animal was arrived at by subtracting from the "live weight" the average weight of the intestines removed from animals of similar weight.

NITROGEN METABOLISM AFTER BURNING.[1] By E. J. Clark, R. A. Peters, and R. J. Rossiter. From the Department of Biochemistry, Oxford.

Changes in nitrogen balance after injury have been described in human patients by Cuthbertson [1930, 1931, 1932, 1934], and in experimental animals by Cuthbertson, McGirr, and Robertson [1939]. Because of the great tissue damage sustained in cases of burns, it seemed of interest to investigate ·more fully the nitrogen metabolism in experimentally burned animals. It is here shown that, after burning, there is a sharp rise in the blood urea (noticeable within one hour), a slight fall in the total plasma protein, and a marked fall in the plasma albumin, giving a decrease in the albumin/globulin ratio. There is, in addition, a rise in the urea output, sharp rise in the creatine excretion, and fall in the excretion of creatinine. With more severe burns, the increase in creatine and decrease in creatinine becomes more marked; there is also a suppression of urea elimination during the first day or so after burning, suggesting some renal impairment. In animals that died as a result of the burning, this decrease in the elimination of urea and creatinine was marked. Weight changes indicate that, at first, there was considerable water retention (a 100-g. rat increased in weight by as much as 14 g. in 24 hours) which gradually decreased over a period of days.

METHODS.

Animals.—Rats (100–150 g.) and rabbits (2–3 kg.) were used in these experiments.

All nitrogen estimations, including blood 'urea, plasma proteins, urine urea, and urine ammonia were determined by the method of Conway and O'Malley [1942]. Creatine and creatinine were determined by the method of Folin [1913].

Methods of Burning.—The rabbits were clipped closely with scissors, anæsthetised with ether and burned by dipping the back (approximately 1/3rd of the body surface) into water at 70° C. for 30 seconds. The rats were anæsthetised with ether and the back (approximately 1/3rd of

113

at which it disappears, but it may be really pathological in the sense that it is a persistent state. There was nothing in the history of this animal which differed from that of the other animals in which the hypertrophy disappeared. It raises the question whether a stimulus which normally produces a physiological hypertrophy may under certain circumstances lead to a pathological hypertrophy. If this is proved to be the case, it would be of interest to determine what are the requisite factors.

SUMMARY.

In a series of animals in which a carotid jugular anastomosis had been patent for three months, the anastomosis was tied off. The hearts of these animals, with one exception, were found almost, if not entirely, to have returned to their normal weight within eight weeks, the auricles reaching normal values before the ventricles. The area of the X-ray cardiac shadow indicated that many of the hearts were twice the normal weight when the anastomosis was tied off.

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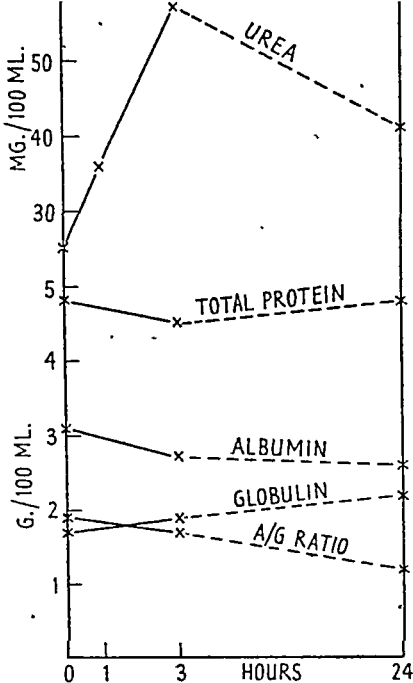
. 1.—Nitrogen constituents in the blood of rats burned at time 0 for 30 seconds
at 80° C. Each figure the mean for 6 or more animals.
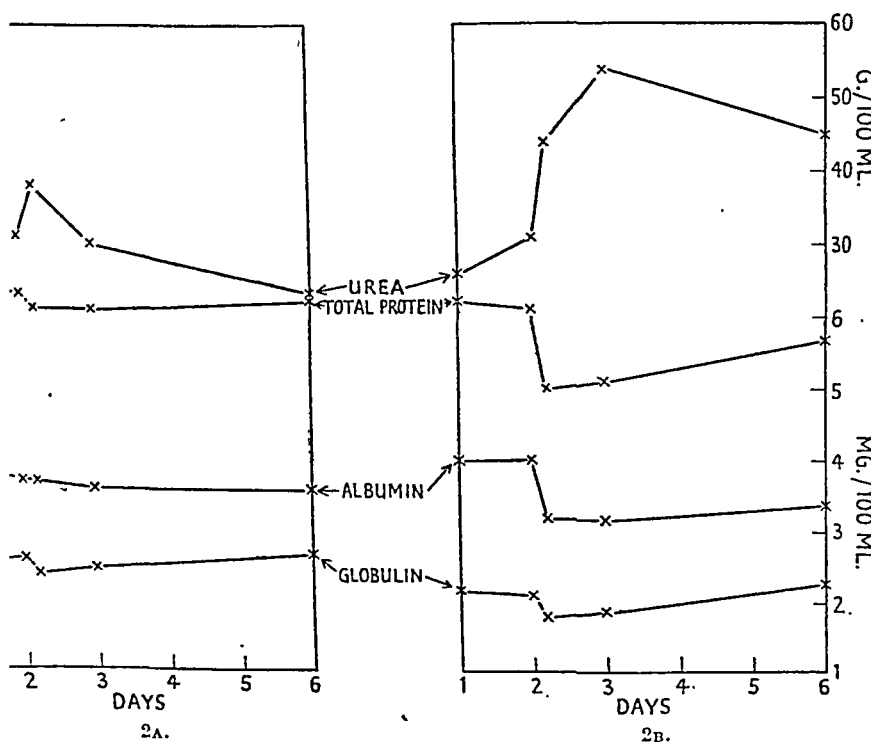
Figs. 2A and 2B.—Nitrogen constituents in blood of rabbits. 2A. Control rabbits
sthetised only on day 2 (mean for 3 animals). 2B. Rabbits burned for 30 seconds
at 70° C. on day 2 (mean for 5 animals).

the body surface) dipped into water at 80° C. for 30 seconds. This method of burning gave damage roughly equivalent to a 1-minute application at 60–65° C. of the burning iron previously described [Leach, Peters, and Rossiter, 1943].[1] Other rats were burned in a similar fashion at 75° C. and 70° C. In all cases control animals were similarly anæsthetised but not burned.

Experimental Arrangements.

For the nitrogen excretion experiments the rats were daily provided with a weighed amount (10 g.) of the following diet:—

Rice starch	.	.	.	70 per cent.
Casein	.	.	.	10 ,,
Salt mixture	.	.	.	5 ,,
Agar agar	.	.	.	2 ,,
Cod-liver oil	.	.	.	3 ,,
Dry yeast	.	.	.	10 ,,

(The salt mixture had the following composition: sodium chloride, 52 g.; magnesium sulphate, 80 g.; sodium phosphate, 102 g.; potassium phosphate, 286 g.; calcium phosphate, 162 g.; calcium lactate, 390 g.; ferric citrate, 35 g.; potassium iodide, 0·2 g.)

The rats were given this diet for a preliminary period of at least 14 days before they were used. Each day the food not consumed at the end of two hours was removed from the cage. This caused a slight fall in body-weight at first, but the rats quickly learned to eat their ration in the given time, and soon it was possible to feed the animals for two hours, and during the remaining 22 hours to collect the urine in metabolism cages. Unlimited water was always given. On this dietary scheme the control animals maintained both weight and nitrogen equilibrium, but they did not grow.

RESULTS.

Blood Urea.—Figs. 1 and 2 show that after burning at 80° C. for 30 seconds the blood urea of rats and rabbits was significantly higher (for rats $t = 3·03$; for $P = 0·01$, $t = 2·78$) at the end of one hour, and markedly higher ($t = 8·41$) at the end of 3 hours, than that of unburned controls. At the end of 24 hours the blood urea of burned animals was still significantly higher (for rats $t = 4·22$) than that of the controls. Details are given in Appendix, Tables I and II. For both the rat and the rabbit, merely anæsthetising gave a slight rise in blood urea, which was not nearly so long lasting as that observed after burning.

[1] In the more severe burns, the urine was occasionally observed to be pink for 1 to 2 days, presumably due to the presence of hæmoglobin (due to local destruction of blood cells at the site of burning). There was no correlation between this appearance and biochemical changes.
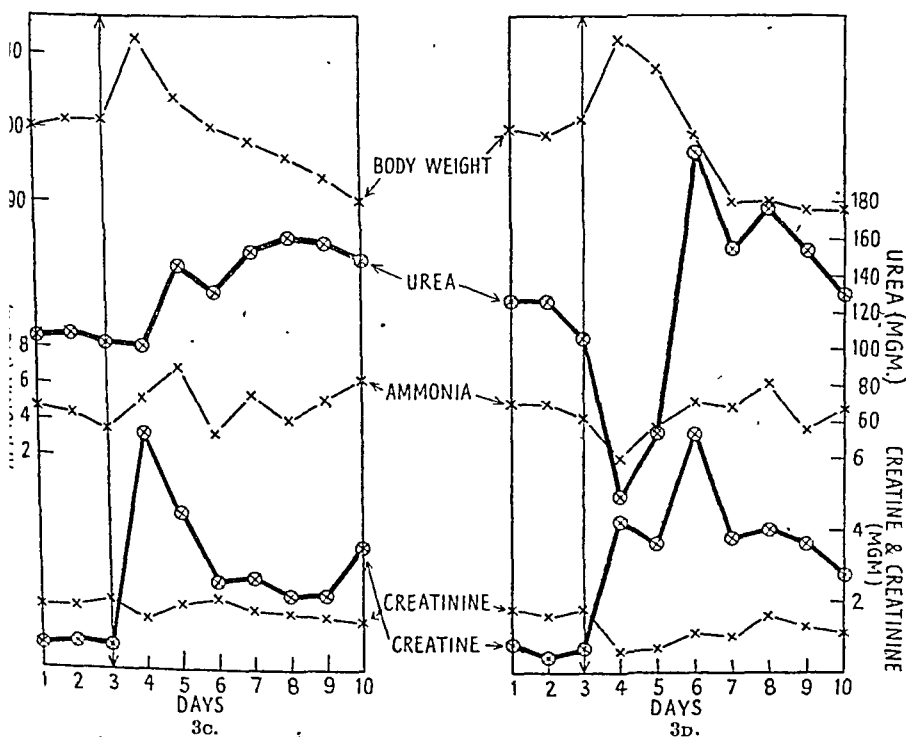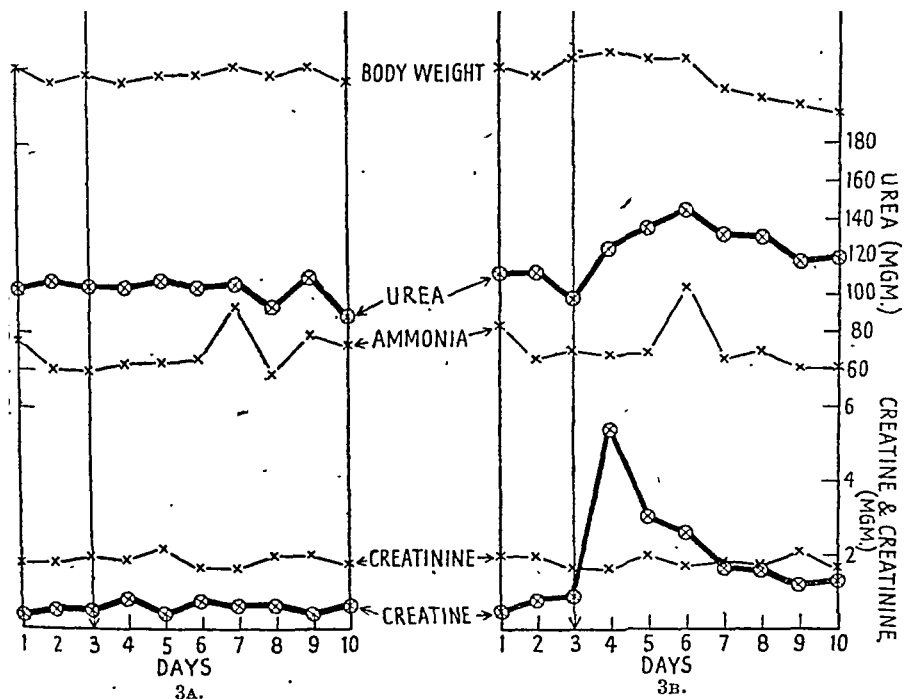
FIGS. 3A, 3B, 3C, and 3D.—Daily urinary nitrogen excretion of rats. All figures refer to a 100-g. rat. 3A. Control rats anæsthetised only after day 3 (mean for 3 animals).

Plasma Proteins.—Figs. 1 and 2 also show that burning caused a slight fall in the total plasma proteins, which in the rat had returned to normal levels within 24 hours, but in the rabbit was still slightly lowered after 4 days. In both species there was a fall in albumin/globulin ratio due to (1) a marked and persistent fall in plasma albumin, and (2) an ultimate rise in globulin. A slight fall at first in globulin in both species was succeeded by a rise after 24 hours in the rat, and after 4 days in the rabbit to above the initial values (for details see Appendix, Tables I and II).

Body-Weight.—The body-weight changes of the rat (figs. 3A, B, C, D) are interesting. Anæsthetising the animal or burning at 70° C. caused but little immediate change in weight, but after burning at 75° C. or 80° C. there was at first a weight increase of the order of 10 per cent. of the body-weight. This can only be accounted for by an increased water retention after the more severe burns. Palpable œdema was much more marked in these animals. Later, there was a gradual fall in body-weight, also seen in those animals burned at 70° C. (which did not show the initial increase), but not observed in the controls that were merely anæsthetised.

Nitrogen Excretion (figs. 3A, B, C, and D).—The tables and figures upon which these are based include data from all rats which lived sufficiently long. These are given, both for 3A, B, and C; the averages presented are from figures which do not appreciably overlap. The one animal which survived after 80° C. is given in fig. 3D, because it showed the interesting fall in N. elimination.

1. *Urea.*—After burning rats at 70° C. there was an immediate increase in the daily excretion of urea. After burning at 75° C. this increase was delayed for one day, and after burning at 80° C. there was a fall in the nitrogen elimination during the first 2 days, followed by a marked rise later. In some cases total nitrogen was estimated in addition to the urea. The results were strikingly parallel and offer no confirmation, in the animal, to the recent claim of Taylor *et al.* [1943] that there is an increased non-urea nitrogen fraction in the urine of burn patients. In every case where both urea and total nitrogen were determined, the urea accounted for over 80 per cent. of the total nitrogen. This is in conformity with the results of Cuthbertson [1930, 1931] who found that, although there was an increase in nitrogen in the urine after operation and injury, urea formed a remarkably constant percentage (about 80 per cent.) of the total.

2. *Creatine.*—The control rats had a slight creatinuria, thus confirming the previous observations of Cuthbertson *et al.* [1939]. After burning there was a dramatic rise in the creatine output, and, in general, the more severe the burn the greater was the amount eliminated. It is noteworthy that with animals burned at 75° C. on the first day, when there was no rise in urea elimination, there was an increased creatine

after plasmapheresis there is a negative nitrogen balance [Kerr et al., 1918]. The same is, in general, true for anhydræmia produced by other means; the relevant facts have been well summarised by Marriott [1923], and for gastroduodenal hæmorrhage by Black [1942]. After burning, besides water loss, there is the additional complication of loss of albumin into the burned area, causing an abnormality of the plasma protein level and of the albumin/globulin ratio. The diversity of conditions, whose sole common factor is anhydræmia, under which these changes in nitrogen metabolism are observed, suggests that parallel with the mobilisation of water from the tissues there is a co-incident mobilisation of protein stores. But the conditions are complex; there is no certainty yet that the changes in tissue permeability do not liberate other factors of importance in the general picture. Further, even if the changes in nitrogen excretion are initiated by the anhydræmia, their long persistence has to be explained. Present information suggests that kidney damage does not explain the raised blood urea so often present in human burns [Taylor, Levenson, Davidson, and Adams, 1943].

Fatal Burns.—During the course of these experiments a number of the animals burned at 80° C. and a few burned at 75° C. (not recorded in the data presented) died. Some of those burned at 75° C. survived for 24 hours, and a study of their nitrogen metabolism for the 24-hour period immediately following the burn is given in figs. 4A and 4B, details being given in Appendix, Tables IVA and IVB. It is seen that there was a rise in creatine excretion, but a marked drop in urea and creatinine elimination.

Thus, burns at 75° C. are followed by a slight diminution in urea and creatinine excretion for one day, a burn at 80° C. produces similar, though greater, changes for 2 days, and the same changes are observed in animals that have died as a result of burning. This can be interpreted as evidence in favour of renal failure. Since histological damage to the kidney is but slight, it seems probable that this damage is associated with the reduction in plasma volume known to occur after burns [Schievers, 1936; Lambret and Driessens, 1937; Keeley *et al.*, 1939; Black 1940 *a*]. That nitrogen retention is a factor contributing to the death of these animals is a point which must claim attention. In all the animals which were investigated and subsequently died, there was this marked retention of nitrogen. Also, animals killed in a moribund condition invariably had a high blood urea—in one case over 300 mg./100 ml.

Practical Applications.—A persisting negative nitrogen balance after burning is a characteristic feature of this work. It is of the order of 50 mg. per day for a considerable number of days. This is equivalent to 500 mg./kg. or 35 g. for an average man. This figure is slightly less than that reported by Taylor *et al.* [1943] for man, and slightly greater than the negative balance described by Lucido [1940] and Cuthbertson

excretion.   The same was true during the 2 days of nitrogen retention which followed burning at 80° C.

3. *Creatinine.*—As would be expected, the creatinine output of the control animals was fairly constant.   This was also true for animals which had been burned at 70° C.   The day following a burn at 75° C. there was a slight fall in the creatinine elimination, and the 2 days following a burn at 80° C. the fall was marked.

4. *Ammonia.*—The elimination of ammonia was somewhat more variable than that of the other substances estimated.   After burning there was no constant change, although in the case of a burn at 80° C. there was less ammonia eliminated during the days immediately after burning, when urea and creatinine elimination was suppressed.

Details of these experiments are given in Appendix, Tables IIIA, IIIB, IIIc, and IIID.

DISCUSSION.

The high non-protein nitrogen that occurs in the blood of experimentally burned animals has previously been reported by Beard and Blalock [1931] and Lowden *et al.* [1939].   Davidson [1925], Beck and Powers [1926], McIver [1933], Wilson and Stewart [1939], and Lucido [1940] have all described either an increased blood non-protein nitrogen or increased blood urea in human burned patients.   Davidson and Matthew [1927], McIver [1933], Weiner *et al.* [1936], Lombard and Montpellier [1937], Elkinton *et al.* [1940], Black [1940 *a*], and Lucido [1940] have reported a decrease in total plasma proteins, while some of the authors [*e.g.* Davidson and Matthew, 1927] have found an increase in the plasma globulin.   Similar changes have been found in experimental animals [Simonart, 1930; Schievers, 1936; Trusler *et al.*, 1939]. Besides the observations of Taylor *et al.* [1943], Lucido [1940] has also reported an increased nitrogen excretion in a patient suffering from severe burns, and this was observed early in the war by Black [1940] (personal communication).

Source of Eliminated Nitrogen.—The source of the eliminated nitrogen is obviously of some theoretical interest.   Cuthbertson *et al.* [1939] favour the view that the excess nitrogen excreted by rats after experimental injury does not come from the site of local damage, and their arguments probably apply to the experiments in this report.

As was thought by Underhill, Fisk, and Kapsinow [1930], the early picture presented in "burn" cases is largely one of decreased blood volume and anhydræmia.   The question is whether the changes in nitrogen metabolism are due to this alone.   Several reports suggest this.   After severe hæmorrhage there is a similar sequence of azotæmia [Taylor and Lewis, 1915; Vallery-Radot *et al.*, 1935; Kaump and Parsons, 1940; Black, 1940 *b*], followed by a negative nitrogen balance [Hawk and Gies, 1904; Buell, 1919; Stewart and Rourke, 1934], and

shown to occur after experimental injury [Cuthbertson *et al.*, 1939]. It is intended to report upon the problem of dietary supplements when further work in progress in this laboratory is completed.

SUMMARY.

Blood and urinary changes in nitrogen compounds in animals after burning at 70°, 75°, and 80° C. for 30 seconds have been compared with anæsthetised controls.

1. *Blood Changes.*—Within an hour after burning there commenced an increase in blood urea in both rats and rabbits, present in the latter four days later. There was also a fall in the total plasma protein and a persistent fall in the plasma albumin. Though falling slightly at first, the globulin levels rose; this caused a progressive fall in the albumin/globulin ratio.

2. *Urinary Changes.*—In rats upon constant diet the following changes occurred with increasing severity of the burn, namely:

(*a*) An initial rise in weight (due to water retention), followed by a fall in weight.

(*b*) Creatinuria.

(*c*) Increased urea elimination; except for early nitrogen retention in the more severe cases for 1 to 2 days. The latter was also a constant feature of rats dying from burns and indicates some form of kidney disturbance.

No constant changes took place in ammonia excretion or in creatinine output, though the latter fell for 1 to 2 days in the more severe burns.

3. The early changes may be explained by the decreased blood volume (shock). The later changes, which are important clinically, indicate a protein mobilisation.

4. As practical measures suggested by the above, carbohydrates should be given during the period of shock, and high protein diets later.

[1930, 1931, 1932, 1934] for man after injury. The nutritional signifi-
cance of these facts is obvious. The loss, which is equivalent to over
200 g. protein per day, must ultimately be made good, and since low
plasma proteins are associated with delayed wound healing [Clark,
1919; Harvey and Howes, 1930], the plasma proteins must be restored
as soon as possible. This underlines the need for increased protein
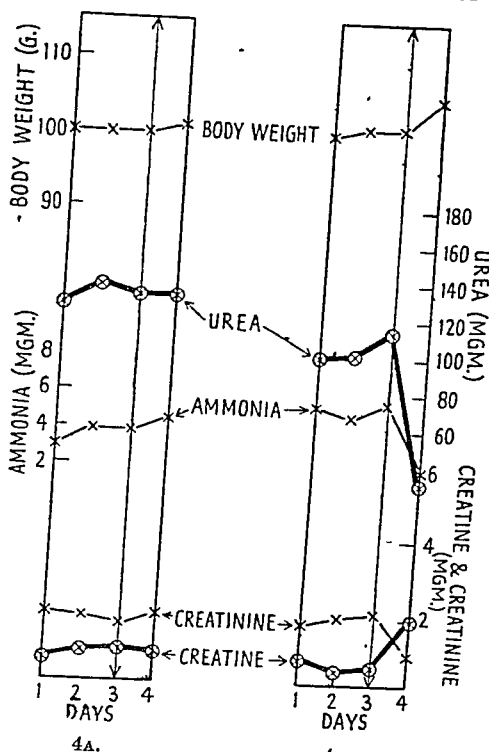
FIGS. 4A and 4B.—Daily urinary nitrogen excretion of rats. All figures refer to a 100-g. rat. 4A. Control rats (mean for 5 animals). 4B. Rats burned for 30 seconds at 75° C. after day 3 and which died after day 4 (mean for 2 animals).

rations and diets of high calorific value for patients during the healing
period. Already in 1936, Cuthbertson found that the ingestion of
diets rich in first-class protein and of high calorific value decreased the
loss of nitrogen that occurs after injury; but since there was no change
in the nitrogen excretion at the height of the catabolic disturbance
immediately succeeding the injury, it seems that a high protein diet is
unnecessary in the period of nitrogen retention (corresponding roughly
with the period of shock). Upon theoretical grounds during this time
extra carbohydrate should be given, for, firstly, there will be a depletion
of the carbohydrate reserves [Clark and Rossiter, 1944], and, secondly,
carbohydrate may exert a valuable protein sparing effect, as has been

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<div align="center">APPENDIX.</div>

TABLE I.—NITROGEN CONSTITUENTS IN THE BLOOD OF RATS.

Treatment.	Blood urea.		Plasma proteins.		
	No. animals.	Mg./100 ml. (± S.E. mean).	No. animals.	Total protein (± S.E. mean).	Albumin (± S.E. mean).
Control (no treatment)	21	25 ± 2	12	4·8 ± 0·1	3·1 ± 0·1
Burned (30 seconds at 80° C.). Sample 3 hours later.	9	57 ± 4	3	4·5 ± 0·1	2·7 ± 0·3
Burned (as above). Sample 24 hours later.	13	41 ± 4	12	4·8 ± 0·1	2·6 ± 0·1
Burned (as above). Sample 1 hour later.	6	36 ± 3			
Anæsthetised only. Sample 1 hour later.	6	26 ± 3			
Anæsthetised only. Sample 3 hours later.	6	37 ± 9			

TABLE IIA.—NITROGEN CONSTITUENTS IN THE BLOOD OF CONTROL RABBITS ANÆSTHETISED ONLY.

Rabbit No.		Day before anæsthetic.	Day of anæsthetic.	4 hours after anæsthetic.	Day after anæsthetic.	4 days after anæsthetic.
B.	Blood urea (mg./100 ml.)	32	24	39	28	24
	Total plasma protein (g./100 ml.)	6·1	6·0	5·6	5·8	5·9
	Plasma albumin (g./100 ml.)	3·9	3·5	3·7	3·6	3·3
E.	Blood urea (mg./100 ml.)	30	44	49	41	22
	Total plasma protein (g./100 ml.)	6·9	7·1	6·8	6·8	6·7
	Plasma albumin (g./100 ml.)	3·9	4·0	3·9	3·7	3·7
J.	Blood urea (mg./100 ml.)	21	26	27	21	24
	Total plasma protein (g./100 ml.)	6·1	5·9	5·8	5·6	6·2
	Plasma albumin (g./100 ml.)	3·8	3·7	3·5	3·4	3·9

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TABLE IIIB.—DAILY URINARY NITROGEN EXCRETION OF RATS BURNED FOR 30 SECONDS AT 70° C. AFTER DAY 3.

All Figures refer to a 100-g. Rat.

	Day No.	Before burning.			After burning.						
		1.	2..	3.	4.	5.	6.	7.	8.	9.	10.
Rat O.	Body-weight (g.) .	100	100	102	101	100	97	94	92	91	88
	Urea N. (mg.) .	117	97	102	125	159	155	138	145	117	124
	Ammonia N. (mg.)	6·6	6·6	5·6	3·5	8·6	20·7	7·4	7·4	4·1	6·1
	Creatinine N. (mg.)	2·3	1·8	1·7	1·7	1·8	1·7	1·4	1·9	2·5	1·4
	Creatine N. (mg.) .	0·6	0·4	0·3	3·7	3·0	2·1	1·4	2·1	0·5	0·2
Rat Q.	Body-weight (g.) .	100	102	102	107	109	107	102	103	102	102
	Urea N. (mg.) .	93	106	86	113	120	132	122	129	135	141
	Ammonia N. (mg.)	8·0	3·4	6·6	5·1	2·7	3·4	3·3	3·4	3·8	3·3
	Creatinine N. (mg.)	1·9	2·3	1·6	1·6	2·1	1·9	2·2	1·7	2·0	1·8
	Creatine N. (mg.) .	0·2	0·5	2·0	4·7	3·0	2·8	1·9	2·2	3·0	3·4
Rat S.	Body-weight (g.) .	100	96	99	97	97	98	96	94	93	93
	Urea N. (mg.) .	123	130	107	133	127	145	133	117	99	93
	Ammonia N. (mg.)	4·5	3·4	3·2	5·9	3·6	4·1	3·0	4·3	4·2	2·9
	Creatinine N. (mg.)	1·7	1·8	1·9	1·8	2·0	1·5	1·7	1·6	1·8	1·8
	Creatine N. (mg.) .	0·8	1·6	0·5	7·7	3·0	2·9	1·9	0·9	0·2	0·2

TABLE IIIC.—DAILY URINARY NITROGEN EXCRETION OF RATS BURNED FOR 30 SECONDS AT 75° C. AFTER DAY 3.

All Figures refer to a 100-g. Rat.

	Day No.	Before burning.			After burning.						
		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
Rat G.	Body-weight (g.) .	100	101	102	109	107	105	105	104	100	
	Urea N. (mg.) .	138	139	120	131	121	140	152	150	156	
	Ammonia N. (mg.)	5·4	4·7	3·3	4·1	2·2	2·4	7·1	2·6	4·1	
	Creatinine N. (mg.)	1·8	1·9	1·9	1·5	1·8	2·4	1·9	1·7	1·7	
	Creatine N. (mg.) .	1·5	1·3	0·7	7·4	3·3	1·9	2·2	0·6	0·5	
Rat N.	Body-weight (g.) .	100	100	100	114	106	100	97	94	94	88
	Urea N. (mg.) .	101	105	92	45	194	117	151	156	133	127
	Ammonia N. (mg.)	5·8	6·0	4·9	3·8	10·7	4·2	6·7	6·3	6·1	5·5
	Creatinine N. (mg.)	1·9	1·9	1·6	1·1	1·9	2·0	1·7	1·5	1·4	1·2
	Creatine N. (mg.) .	0·3	0·6	0·6	5·2	5·5	1·9	2·4	2·9	2·6	2·7
Rat R.	Body-weight (g.) .	100	102	101	112	99	96	93	89	86	84
	Urea N. (mg.) .	75	76	95	124	121	134	154	174	181	167
	Ammonia N. (mg.)	2·9	2·4	2·3	7·7	7·8	3·1	2·4	3·2	5·0	6·8
	Creatinine N. (mg.)	1·8	1·6	2·4	1·8	2·0	1·7	1·5	1·5	1·3	1·6
	Creatine N. (mg.) .	0·4	0·4	0·7	7·1	4·3	3·8	3·2	2·7	3·2	4·2

TABLE IIB.—NITROGEN CONSTITUENTS IN THE BLOOD OF RABBITS BURNED
30 SECONDS AT 70° C.

Rabbit No.		Day before burning.	Day of burning.	4 hours after burning.	Day after burning.	4 days after burning.
A.	Blood urea (mg./100 ml.)	26	34	50	38	41
	Total plasma protein (g./100 ml.)	7·3	7·8	6·5	6·3	6·7
	Plasma albumin (g./100 ml.)	3·5	3·9	3·5	2·8	3·4
C.	Blood urea (mg./100 ml.)	25	35	51	49	28
	Total plasma protein (g./100 ml.)	6·1	5·5	4·8	4·8	5·0
	Plasma albumin (g./100 ml.)	4·2	4·1	3·1	3·1	3·4
K.	Blood urea (mg./100 ml.)	26	32	39	42	37
	Total plasma protein (g./100 ml.)	5·5	5·4	4·4	4·3	5·2
	Plasma albumin (g./100 ml.)	4·2	4·0	3·1	3·1	3·4
N.	Blood urea (mg./100 ml.)	25	27	48	87	64
	Total plasma protein (g./100 ml.)	6·1	6·2	5·3	5·3	6·3
	Plasma albumin (g./100 ml.)	4·2	4·2	3·4	3·5	3·8
U.	Blood urea (mg./100 ml.)	29	25	32	56	55
	Total plasma protein (g./100 ml.)	6·0	5·8	4·1	4·8	5·4
	Plasma albumin (g./100 ml.)	3·9	3·9	2·8	2·9	3·3

TABLE IIIA.—DAILY URINARY NITROGEN EXCRETION OF RATS ANÆSTHETISED
AFTER DAY 3.

All Figures refer to a 100-g. Rat.

	Day No.	Before anæsthesia.			After anæsthesia.						
		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
Rat C.	Body-weight (g.)	100	99	99	100	101	101	101	101	103	100
	Urea N. (mg.)	102	100	95	95	100	89	95	94	116	77
	Ammonia N. (mg.)	4·0	2·7	3·9	3·1	3·6	3·7	4·0	3·7	4·1	2·7
	Creatinine N. (mg.)	1·4	1·5	1·8	1·5	1·7	1·2	1·4	2·1	1·7	1·3
	Creatine N. (mg.)	0·4	0·4	0·2	0·2	0·2	0·8	0·9	0·6	0·7	1·0
Rat T.	Body-weight (g.)	100	98	100	98	100	101	100	99	100	100
	Urea N. (mg.)	104	112	98	112	112	111	117	83	107	97
	Ammonia N. (mg.)	5·4	5·3	4·2	5·6	5·2	5·8	5·5	3·9	6·5	6·7
	Creatinine N. (mg.)	2·0	2·0	2·2	2·2	2·2	1·8	1·9	1·9	2·2	2·4
	Creatine N. (mg.)	0·2	0·4	0·4	1·1	0·8	0·7	0·8	0·7	0·2	0·3
Rat U.	Body-weight (g.)	100	98	99	97	97	95	98	96	97	95
	Urea N. (mg.)	104	109	118	104	111	108	107	96	103	89
	Ammonia N. (mg.)	7·1	4·0	3·7	4·2	4·1	4·1	12·5	3·4	6·7	6·5
	Creatinine N. (mg.)	2·2	1·9	2·1	1·9	2·7	2·1	1·9	2·0	2·2	1·7
	Creatine N. (mg.)	0·5	0·8	0·8	1·1	0·2	0·7	0·2	0·5	0·2	0·6

TABLE IVʙ.—Daily Urinary Nitrogen Excretion of Rats Burned for 30 Seconds at 75° C. after Day 3, and which Died after Day 4.

All Figures refer to a 100-g. Rat.

	Day No.	1.	2.	3.	4.
Rat L.	Body-weight (g.)	100	101	101	104
	Urea N. (mg.)	86	·95	102	40
	Ammonia N. (mg.)	6·5	5·0	5·9	2·0
	Creatinine N. (mg.)	1·5	1·6	1·7	1·0
	Creatine N. (mg.)	0·2	0·4	0·3	1·9
Rat M.	Body-weight (g.)	100	104	102	109
	Urea N. (mg.)	114	107	126	22
	Ammonia N. (mg.)	4·0	4·5	5·0	1·0
	Creatinine N. (mg.)	1·9	2·2	2·3	0·7
	Creatine N. (mg.)	1·2	0·4	0·7	1·6

TABLE IIId.—DAILY URINARY NITROGEN EXCRETION OF RAT BURNED FOR
30 SECONDS AT 80° C. AFTER DAY 3.

All Figures refer to a 100-g. Rat.

	Day No.	Before burning.			After burning.						
		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
Rat B.	Body-weight (g.) .	100	99	101	112	108	99	90	90	89	89
	Urea N. (mg.) .	126	125	106	19	53	207	156	177	153	129
	Ammonia N. (mg.)	5·0	4·9	4·2	2·0	3·6	5·1	4·9	6·1	3·5	4·8
	Creatinine N. (mg.)	1·8	1·6	1·8	0·6	0·7	1·1	1·0	1·6	1·3	1·1
	Creatine N. (mg.) .	0·8	0·4	0·7	4·2	3·6	6·7	3·8	4·0	3·6	2·7

TABLE IVA.—DAILY URINARY NITROGEN EXCRETION OF CONTROL RATS.

All Figures refer to a 100-g. Rat.

	Day No.	1.	2.	3.	4.
Rat A.	Body-weight (g.) .	100	101	101	100
	Urea N. (mg.)	108	131	129	115
	Ammonia N. (mg.)	3·6	4·0	3·2	4·3
	Creatinine N. (mg.)	1·6	1·8	1·6	1·6
	Creatine N. (mg.) .	0·9	1·0	0·4	0·7
Rat. D.	Body-weight (g.) .	100	103	101	103
	Urea N. (mg.)	118	143	133	144
	Ammonia N. (mg.)	2·8	4·0	4·7	5·3
	Creatinine N. (mg.)	1·9	1·7	1·6	1·9
	Creatine N. (mg.) .	0·7	1·2	1·5	1·0
Rat E.	Body-weight (g.) .	100	100	103	103
	Urea N. (mg.)	107	114	116	105
	Ammonia N. (mg.)	2·9	3·9	3·8	5·3
	Creatinine N. (mg.)	1·8	1·7	1·2	1·8
	Creatine N. (mg.) .	0·4	0·6	1·3	0·7
Rat F.	Body-weight (g.) .	100	100	99	100
	Urea N. (mg.)	118	127	122	131
	Ammonia N. (mg.)	3·4	4·4	4·6	4·4
	Creatinine N. (mg.)	1·8	1·8	1·4	2·1
	Creatine N. (mg.) .	0·6	0·6	1·0	0·9
Rat I.	Body-weight (g.) .	100	95	96	98
	Urea N. (mg.)	180	171	161	166
	Ammonia N. (mg.)	2·2	3·3	2·6	3·3
	Creatinine N. (mg.)	2·2	1·8	2·1	2·1
	Creatine N. (mg.) .	0·3	0·7	0·3	0·6

INTERCELLULAR PLASMA IN THE CENTRIFUGED ERYTHRO-CYTES OF NORMAL HUMAN BLOOD. By MONTAGUE MAIZELS. From the South-Eastern Emergency Blood Supply Depot, Maidstone.

WHEN investigating the chemical and physical properties of erythrocytes it is often necessary to have a measure of the cell mass examined. So too, when measuring the volume of the circulating blood in the intact animal, the proportion of cells relative to blood must be known. Such methods all depend on the separation of cells from plasma by centrifuging, but since deformation of cells does not permit of perfect packing, some plasma always persists in the centrifuged cell mass so that the absolute volume of cells cannot be obtained. It has usually been assumed that the amount of intercellular plasma is relatively small and that for most purposes it can be ignored. This view is supported by the results of many investigators whose work will be reviewed later. Recently, however, Chapin and Ross [1942] have stated that cell volume as measured by the hæmatocrite is about 8·5 per cent. too high. If this figure were correct, measurements of circulating blood volume would be greatly affected, for in normals the error would be + 8 per cent., while in severe anæmia it might only be + 1 per cent. Apart from these effects, due to simple quantitative changes in the blood cells, qualitative changes in disease might lead to altered packing and hence to variations in intercellular plasma. If normal intercellular plasma were, in fact, itself quite small, then probably variations due to disease would for practical purposes be negligible. But if the true normal value were between 8 and 9 per cent., as Chapin and Ross suggest, then pathological variations might well be significant and all data depending on hæmatocrite determinations open to doubt. Since, then, intercellular plasma has an important bearing on the physiology of the circulation and on the physico-chemical investigation of the blood, it seemed desirable to redetermine its value and define its limits as far as possible.

Most investigators have used an indirect method of measurement, in which a known amount of dye is added to a measured volume of blood. The colour of the separated plasma is then compared with that of the same amount of dye added to known volumes of plasma,

removed with a fine angle-tipped pipette. The volume of cells—about fifty divisions or 1 ml.—was now read, 2 ml. saline added and the cells resuspended by gentle but thorough mixing, without shaking. The tube was then recentrifuged for thirty minutes, when the volume of cells was found to be practically unaltered. Protein in 1 ml. of the supernatant was now compared with that in 1 ml. of the dilute standard. This was done colorimetrically by the method of Wu and Ling [1927] and in a few instances by wet-ashing in sulphuric acid, and Nesslerization.

(B) Here 0·5 ml. Evans blue in saline (0·25 per cent.) was added to 2·7 ml. blood. After mixing, centrifuging, and washing in the usual way, the cells were resuspended in 2 ml. saline and again centrifuged. The blue-tinted saline was then compared with the original supernatant plasma-dye mixture diluted one hundred times. This method, in contrast to (A), does not give high results, should manipulation produce slight hæmalysis.

Ostwald pipettes were used for measuring and all volumetric apparatus was calibrated. The maximum errors in the comparator tube measurements and in the colour readings were ± 2·5 per cent. in each case, giving a total of ± 5 per cent. In addition, imperfect washing would give high results in both methods, while hæmolysis due to manipulation would give high results in method (A). In practice duplicate experiments agreed within ± 8 per cent.; for present purposes, this difference is negligible.

RESULTS.

Results are shown in Table I.

TABLE I.—INTERCELLULAR PLASMA IN HUMAN ERYTHROCYTES CENTRIFUGED FOR 30 MINS. AT 3000 R.P.M.

	Citrated blood.	Heparinised blood.		Heparinised blood, buffy coat retained.
Method . . .	A.	A.	B.	A. or B.
Number of observations	16	12	7	9
Average, and standard deviation . .	2·28 ± 0·16	2·24 ± 0·19	2·16 ± 0·26	2·74 ± 0·31

In the case of bloods which had had their buffy coats removed, intercellular plasma varied between 2 and 2·5 per cent., irrespective of the anticoagulant and method used. Methods depending on protein colour reactions gave rather higher results than the simple dye technique, possibly because the saline used for extracting the erythrocytes contained traces of hæmolysed cells not present in the diluted standard. Still higher results were obtained by Nesslerization: in three experiments, Wu and Ling's method gave 2·27, 2·32, and 2·23 per cent., corresponding with 2·36, 2·46, and 2·58 per cent. on Nesslerization. This may be due

and from the comparison the amount of plasma and hence the true volume of cells in the original sample of blood may be calculated. The difference between this and the hæmatocrite volume equals plasma trapped between the cells. The dye selected is such as to penetrate the erythrocytes little or not at all. The disadvantage of the method lies in the high degree of accuracy required in spite of the difficulty of comparing the colour of dyes dissolved in plasma—a difficulty which led Harington, Pochin, and Squire [1940] to modify the Evans blue method for determining the circulating blood volume, by extracting the dye from plasma with butyl alcohol. It is therefore surprising that the majority of workers on true cell volume have preferred the indirect method, although an error of 1 per cent. in the colorimeter readings of plasma colours corresponds to an error of 35 per cent. or more in the figure for intercellular plasma. Such errors may well account for the variations recorded by different observers and the wide range encountered in individual series.

In the direct method use is made of some suitable indicating substance naturally present or added to blood. The blood is centrifuged, supernatant plasma washed away, the erythrocytes resuspended in saline and the suspension recentrifuged. Plasma occluded between cells after the first centrifuging remains in the supernatant saline after the second centrifuging and the indicator substance therein is compared with an appropriate dilution of the same indicator. From the data so obtained, the amount of intercellular plasma is calculated. The method presents no special difficulties beyond that of washing away supernatant plasma without disturbing the centrifuged cells. Errors in measurements are not magnified in the results, and an error of 1 per cent. in the colorimeter reading produces an error of 1 per cent. in the value for intercellular plasma. In spite of this the direct method has rarely been used. It had, however, a special value for the present writer, in that colorimetric readings could be made with an ordinary direct vision colorimeter.

<center>METHODS.</center>

Two variations were used: (A) Heparinised or citrated human blood was centrifuged for thirty minutes at 3000 r.p.m. in graduated hæmoglobinometer tubes of about 7 mm. bore. The radius of the centrifuge head was 15 cm. Supernatant plasma was removed with a teat and capillary pipette, and, diluted one hundred times, constituted the standard of comparison. The "buffy coat" was left *in situ* and the tube carefully washed out twice with saline. The buffy coat was next removed and a third saline wash given without disturbing the subjacent cells. This saline and the upper layer of centrifuged cells was then drawn off and the *tube recentrifuged for five minutes, after* which any saline that had drained down the sides of the tube was

is read and the cells suspended in 1 ml. saline. After recentrifuging, protein was estimated by the method of Wu and Ling. Results gave 2·75, 3·05, 3·54, and 3·71 for intercellular plasma. It had been expected that the figures would be less and not more than those for narrow tubes, and it was thought that resuspending cells from narrow tubes by means of capillary pipettes had led to hæmolysis. Accordingly, the experiments were repeated by the direct Evans blue method, saline washes from the cells of undyed blood being added to the standard solution so as to equalise coloration due to hæmolysis in all samples. By this method, intercellular plasma was found to be 1·70 and 1·62 for cells with the buffy coat removed and 1·90 and 1·87 for cells retaining the buffy coat.

In view of the number of indirect experiments carried out by other observers, a few were included in the present series. The first three differed from those of Chapin and Ross [1942] in that a concentrated solution of dye was used as recommended by Shohl and Hunter [1941], so that both unknown and standard could be diluted one hundred times before being compared. In this way interference by plasma proteins, etc., was minimised. A direct vision colorimeter was quite unsuited to this work and use was made of a Hilger "Spekker" absorptiometer which was temporarily available in the pathology department of the Kent County Council. The following results were obtained: (A) 1·56, 1·28, 1·70, 1·70 per cent.; (B) 1·45 and 2·68 per cent.; (C) 2·68 and 3·62 per cent. An attempt was made to follow Chapin and Ross's dilute dye technique, but this was frustrated by the lipæmic condition of the plasma. In another experiment, with a less lipæmic plasma, so much absorbtion was due to the plasma itself that a 90 per cent. standard could hardly be distinguished from the 100 per cent. It was felt that this method was inferior to the preceding one of Shohl and Hunter [1941].

DISCUSSION.

The volume of cells in centrifuged blood depends on the size of the erythrocytes, the amount of intercellular plasma and the degree of cell compression. At low speeds the intercellular plasma is high and compression low; at high speeds conditions are reversed. According to Ponder and Saslow [1930], therefore, there is no such thing as an absolute hæmatocrite value. But the practical question remains: whether a significant amount of intercellular plasma remains after centrifuging is carried out in the usual way—at 3000 r.p.m.?

Of the large group of those investigating this question by the indirect technique, Keith, Rowntree, and Geraghty [1915] give + 3·5, 0, and + 0·5 per cent. Hooper, Smith, Belt, and Whipple [1920] give – 2 and + 4·5 per cent., negative values indicating that the centrifuge value is less than the dye value.

to the fact that the nitrogen/tyrosine ratio in hæmoglobin is higher in hæmoglobin than in plasma proteins [Plimmer, 1917], so that hæmolysis would give a greater increase in colour on Nesslerization than on treatment with the reagents used in the method of Wu and Ling.

Details of technique have received further consideration: washing could be carried out without disturbing erythrocytes and consistency of results suggested that no plasma was left behind, nor any extracted from the packed cells. This was confirmed by the method of Shohl and Hunter [1941]. Blood containing dye was centrifuged in narrow test-tubes waisted at the junction of the middle and lower thirds. Supernatant plasma was removed, the tube cut at the constriction and the upper portion containing buffy coat and a small amount of packed cells discarded. In this way cells were obtained free of supernatant plasma without washing. They were transferred in saline from the cut bulb to a test-tube and the suspension examined in the usual way. The bulb was finally calibrated, and results showed that intercellular plasma derived from erythrocytes prepared in this way was the same as when cells were washed in the usual manner—1·87 and 1·95 per cent. in the former and 1·91 and 1·93 per cent. in the latter.

In the preceding experiments the "buffy coat" was removed before extraction with saline. Table I shows that if the buffy coat is retained, intercellular plasma appears to be consistently high. It is possible that this may be due in part to a higher plasma content in the buffy coat and superficial cell layers, but it was thought that the chief factor was trapping of plasma below the buffy coat as a result of wrinkling of that layer during the first saline wash.

There was no evidence that repeated extraction of packed cells yielded significantly higher figures for intercellular plasma. In one blood this was 2·12 per cent. after a single saline wash, and the same figure was obtained after three washes. In a second experiment using the dye method, a first extraction gave 2·4 per cent., while three extractions yielded a total of 2·47 per cent. Variations in the amount of dye added to blood did not affect the figures for intercellular plasma appreciably. In the case of one blood, the initial contents of dye in the plasma from three samples was 130, 32·5, and 16·25 mg. per cent.; the corresponding figures for intercellular plasma were 1·93, 1·99, and 2·01 per cent. The fact that increased dye is not taken up by erythrocytes as the external concentration is increased suggests that little if any adsorbtion occurs, an observation which agrees with the conclusions of Gregersen and Schiro [1938].

Hitherto, experiments have been carried out with tubes of 7 mm. bore; the next series were done with tubes of only 3 mm. bore. After centrifuging blood in tubes drawn out into sealed, graduated, and calibrated capillaries of 3 mm. bore, the volume of cells, about 0·1 ml.,

decrease in plasma protein the true plasma volume is calculated from Chapin and Ross's equation.   Thus plasma volume =

$$\text{saline added} \times \text{mg. protein per ml. diluted plasma}$$
$$\overline{\text{mg. protein per ml. original plasma} - \text{mg. protein per ml. diluted plasma.}}$$

A single set of figures only is given:

$$\text{true plasma volume} = 1 \times 62 \cdot 00/(73 \cdot 50 - 62 \cdot 00)$$

whence true plasma volume = 53·9 per cent. and true cell volume = 46·1 per cent., which is about 9 per cent. less than the hæmatocrite volume of 50·6 per cent.

Protein was estimated by the falling drop method of Kagan [1938]. According to Kagan this, in 107 experiments, differed from results by the Kjeldahl method by ± 1·8 mg. per ml., with a maximum difference of 4·8 mg.   If this is so, then the above data might have been 62·00 ± 1·8 and 72·50 ± 1·8 mg., and the true cell volume might have been somewhere between 20 and 60 per cent.   It is of course probable that Chapin and Ross's figures were much nearer to the true values than this, but.it is felt nevertheless that while Kagan's method is suited to determining plasma proteins in unrelated samples, it is not adapted to giving small difference values between high protein contents.   On the whole, therefore, it seems likely that, owing to some unexplained factor, the results of Chapin and Ross are too high.

Turning now to results obtained by direct measurement of plasma occluded in centrifuged erythrocytes, there are occasional results recorded here and there: 2 per cent. by Shohl and Hunter [1941] and 3·9 and 3·9 per cent. by Gregersen and Schiro [1938].   Crabtree and Maizels [1937] record a small series of direct determinations averaging 5 per cent. in cells with the buffy coat retained.   It is probable that this figure is too high on account of excessive hæmolysis due to manipulation of cells in narrow tubes.   Oberst [1935] gives an average figure of 3 per cent.   He measured the sodium content of packed cells before and after washing with isotonic potassium chloride solution and assumed that the fall in value after washing was solely due to removal of sodium in the intercellular plasma.   It is probable, however, that some slight leakage of sodium from the cells themselves occurs during washing, in which case intercellular plasma will be slightly less than the 3 per cent. which Oberst gives.

CONCLUSIONS.

If blood is centrifuged in tubes of 7 mm. bore, the intercellular plasma amounts to 2·7 per cent. of packed cells if the buffy coat is retained and to about 2·25 per cent. if the buffy coat is removed.   In narrower tubes the values are slightly less.   This intercellular plasma

Smith [1920] gives +2, -2, -3, -4, and -5 per cent. Ponder and Saslow [1930], using an indirect method originally suggested by Stewart [1899], found that samples of one blood centrifuged for 12 and 20 minutes respectively, gave values for intercellular plasma of +1 and 0 per cent.; another sample of blood at 15 and 30 minutes gave values of +2·5 and -5 per cent. According to Wintrobe [1931] the average value given by the hæmatocrite at 3000 r.p.m. is about the same as that given by the dye method. Gregersen and Schiro [1938] give very variable figures for animal blood; two values for human bloods were about 4 per cent. Shohl and Hunter [1941] investigated the amount of intercellular plasma with strong solutions of Evans blue, diluting the plasma-dye mixtures with saline before examination. Their value and standard deviation is 4·4 ±1·4 per cent. Probably because of inherent defects in the indirect dye method, their experimental error was equivalent to ±12 per cent. of the value for intercellular plasma, although it corresponded with only ±0·4 per cent. in the colorimeter readings. These last results correspond with our own for cells with the "buffy coat retained" in Table I, where the figure is 2·74 per cent. It must be noted, however, that Shohl and Hunter have empirically increased the hæmatocrite by 1·1 per cent. to compensate for failure to include the buffy coat in the actual reading. Had such compensation been made in column 4 of Table I, the value would have been 3·84 per cent., which is only slightly lower than Shohl and Hunter's own figure. It may be noted that in one direct experiment on centrifuged cells with the buffy coat removed, Shohl and Hunter found only 2 per cent. of intercellular plasma, which compares well with 2·28 per cent. in column 2.

In contrast to most of these observations are those of Chapin and Ross [1942], who find that the hæmatocrite is about 8·5 per cent. higher than the true cell volume. They used the indirect dye technique exclusively in several variations, and their results are so consistent that it may be wondered if a constant error is not involved in the method which appears to assume that if allowance is made for absorbtion of light by normal plasma constituents, the dye constant is the same in plasma as in saline. Since, however, Evans dye adsorbs on globulin [Chapin and Ross, 1942], it is possible that the absorbtion of light by dye and globulin combined is not exactly the sum of their separate absorbtions. This possible objection applies with less force to Chapin and Ross's method B, which, however, presents certain other difficulties. It would not apply to Shohl and Hunter who used a heavily dyed plasma, highly diluted with saline, in contrast with Chapin and Ross who used a lightly dyed plasma undiluted with saline. However this may be, Chapin and Ross [1942] find intercellular plasma to be 8·5 per cent. by another method which is the indirect form of one used by Crabtree and Maizels [1937]. 1 ml. saline is added to 10 ml. blood, and from the

THE FATE OF ARSENIC IN THE BODY FOLLOWING TREATMENT OF RABBITS WITH CERTAIN ORGANIC ARSENICALS.

By AVERIL C. CHANCE, T. B. B. CRAWFORD, and G. A. LEVVY. From the Departments of Biochemistry and Pharmacology, University of Edinburgh.

VOEGTLIN and THOMPSON [1922] have suggested that the toxicity of an arsenical is governed to a large extent by its rate of excretion. Phenylarsenoxide has a high toxicity compared with those of arsenicals used in therapeutics. In an effort to determine to what extent this can be attributed to the degree to which the arsenic-containing molecule is retained in the body, and, in particular, in vital organs, rather than to any specific properties not shared by similar arsenicals, organs and excreta of rabbits were analysed following injection with phenylarsenoxide, mapharsen (*m*-amino-*p*-hydroxyphenylarsenoxide) and with the arsonic acid corresponding to each arsenoxide. This work had to be abandoned without completing the full programme originally planned, but it is thought that the results obtained should be put on record. While further information on certain points would be desirable, the main objective has been attained.

A great deal of work has been published on the fate of mapharsen in the body following intravenous injection. The distribution and excretion of arsenic following treatment of dogs with massive doses was studied by Magnuson and Raulston [1941]. Figures for the excretion of mapharsen by dogs have also been given by Gruhzit, Dixon *et al.* [1936]. In rats, the distribution has been described by Wright *et al.* [1937] and the excretion by Voegtlin and Thompson [1922]. Excretion figures for mapharsen in man have been reported by Foerster and his co-workers [1935] and by Henning and Kampmeier [1943]. All figures for the excretion of mapharsen are in agreement in suggesting that this is a fairly rapid process, at least half of the arsenic administered being cleared in a week. In man and the dog, in contrast to the rat, more arsenic was found in the fæces than in the urine.

In a paper received after we had ceased work on this problem, Hogan and Eagle [1944] give an account of a detailed and extensive study of the basis for the varying toxicity of arsenicals. Results are

accounts for the difference observed between the true cell volume and the volume observed on centrifuging.   The difference is so small that it may be neglected for practical purposes.

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THE FATE OF ARSENIC IN THE BODY FOLLOWING TREATMENT OF RABBITS WITH CERTAIN ORGANIC ARSENICALS.

By Averil C. Chance, T. B. B. Crawford, and G. A. Levvy. From the Departments of Biochemistry and Pharmacology, University of Edinburgh.

Voegtlin and Thompson [1922] have suggested that the toxicity of an arsenical is governed to a large extent by its rate of excretion. Phenylarsenoxide has a high toxicity compared with those of arsenicals used in therapeutics. In an effort to determine to what extent this can be attributed to the degree to which the arsenic-containing molecule is retained in the body, and, in particular, in vital organs, rather than to any specific properties not shared by similar arsenicals, organs and excreta of rabbits were analysed following injection with phenylarsenoxide, mapharsen (*m*-amino-*p*-hydroxyphenylarsenoxide) and with the arsonic acid corresponding to each arsenoxide. This work had to be abandoned without completing the full programme originally planned, but it is thought that the results obtained should be put on record. While further information on certain points would be desirable, the main objective has been attained.

A great deal of work has been published on the fate of mapharsen in the body following intravenous injection. The distribution and excretion of arsenic following treatment of dogs with massive doses was studied by Magnuson and Raulston [1941]. Figures for the excretion of mapharsen by dogs have also been given by Gruhzit, Dixon *et al.* [1936]. In rats, the distribution has been described by Wright *et al.* [1937] and the excretion by Voegtlin and Thompson [1922]. Excretion figures for mapharsen in man have been reported by Foerster and his co-workers [1935] and by Henning and Kampmeier [1943]. All figures for the excretion of mapharsen are in agreement in suggesting that this is a fairly rapid process, at least half of the arsenic administered being cleared in a week. In man and the dog, in contrast to the rat, more arsenic was found in the fæces than in the urine.

In a paper received after we had ceased work on this problem, Hogan and Eagle [1944] give an account of a detailed and extensive study of the basis for the varying toxicity of arsenicals. Results are

quoted for the distribution and excretion of a number of compounds injected intravenously into rabbits. Some of the figures refer to compounds with which we worked. The excretion of arsenic in urine and fæces combined was quicker and more complete with mapharsen than with phenylarsenoxide. Injection of median lethal doses of phenylarsonic acid and phenylarsenoxide gave comparable tissue levels once the initial rapid excretion was over, in spite of the sixteen-fold difference in the amount of arsenic injected. As with other arsenicals, mapharsen and phenylarsenoxide were bound by liver and kidney in proportion to their toxicity.

The only other published figures available for the arsenicals dealt with here appear to be those given by Voegtlin and Thompson [1922] for the excretion of m-amino-p-hydroxyphenylarsonic acid in rats. Twenty-four hours after intravenous injection, 89 per cent. of the arsenic was found in the urine and 5 per cent. in the fæces. Other studies on the fate of organic arsenicals in the body deal with arsphenamine and neoarsphenamine [Voegtlin, 1925; Underhill and Dimick, 1928; Kraft et al., 1938].

EXPERIMENTAL.

Before use, phenylarsenoxide was recrystallized from a mixture of benzene and ether, and phenylarsonic acid was recrystallized from water. Solutions of these for injection were prepared by dissolving the solid in sodium hydroxide solution containing sufficient sodium ion to give a concentration in the final volume equivalent to that in 0·9 per cent. NaCl. Before the final dilution, the solution was brought to neutrality with hydrochloric acid, using a glass electrode. Solutions of m-amino-p-hydroxyphenylarsonic acid were made up immediately before use by adding equivalent amounts of $NaHCO_3$ to aqueous suspensions of the solid. In the case of mapharsen, a commercial preparation ("Mapharside," Parke, Davis & Co.) was used. The total arsenic concentrations in the solutions of all the compounds were checked by analysis, and the trivalent arsenic contents were determined by iodine titration. The arsenicals were administered by intravenous injection.

For experiments involving the collection of urine and fæces, male rabbits only were used. They were kept in metal metabolism cages of the usual type. This led to no appreciable error through contamination of the excreta or retention of arsenic by the metal. At the end of each period of collection of excreta, the animal was catheterised and the urine added to that obtained from the washed metabolism cage. In all experiments, heparin (500 I.U.) was injected before killing the animal. The rabbit was then stunned and bled from the carotid arteries. More heparin (ca. 500 I.U.) was added to the blood, and the cells were separated by centrifuging. Organs were cut up into small pieces and aliquots were taken for arsenic determination [Levvy, 1943]

after as much residual blood as possible had been removed by wiping with filter paper. Wherever possible, replicate samples were analysed. No detectable amounts of arsenic were found in the organs or excreta of normal animals.

RESULTS.

Table I summarizes the results of all experiments with the four arsenicals studied. Doses are expressed in terms of elemental arsenic. To adjust for differences in dosage, the figure found for As in each g. of moist tissue was divided by the number of mg. As injected per kg.

TABLE I.—DISTRIBUTION AND EXCRETION OF ARSENIC.

(Tissue figures: μg. As/gm. for dose of 1 mg. As/kg. Excretion figures: μg. As per mg. As injected. "tr": trace. "nd": not done.)

Expt. no.	7.	3.	5.	8.	16.	11.	10.	13.	14.	15.	12.
Arsenical	Phenylarsenoxide.					m-Amino-p-hydroxyphenylarsenoxide.					
Time killed, hrs.	1	1	1	24	168	1	1	24	24	168	144
Dose, mg. As/kg.	0·45	0·89	0·89	0·45	0·45	0·67	3·48	0·44	3·48	0·44	3·48
Wt. of rabbit, kg.	2·80	2·60	2·10	2·05	2·50	2·05	2·50	2·65	2·50	2·95	1·85
Sex	M.	M.	F.	M.	M.	F.	F.	M.	M.	M.	M.
Lung	14·9	11·6	10·0	tr	tr	tr	0·86	nil	0·43	tr	nil
Liver	7·51	5·05	3·81	5·15	1·47	1·64	1·49	tr	0·43	nil	0·41
Kidney	8·97	6·28	6·63	2·11	1·67	2·98	3·51	tr	2·22	nil	0·60
Blood cells	4·77	2·00	1·84	nil	tr	tr	1·58	nil	nd	nil	nil
Plasma	nd	0·01	0·27	nil	tr	tr	nd	nil	nd	nil	nil
Small intestine	2·31	1·60	2·10	nil	0·84	1·79	1·98	nil	0·40	nil	0·12
Muscle (vol.)	0·73	0·34	0·47	tr	0·76	0·30	0·26	nil	tr	tr	0·07
Brain	tr	nil	nd	nd	nil	nil	nil	nil	nil	tr	nil
Heart	2·07	1·19	2·92	nil·	nil	nil	0·46	nil	tr	nil	nil
Bone (rib) + marrow	nil	nd	nd	nil	tr	nd	nd	nil	0·20	0·23	nil
Bone marrow	tr	nil	tr	nil	nil	tr	tr	nil	tr	nil	nil
Spleen	tr	tr	tr	nil	tr	nil	nil	nil	nil	nil	nil
Skin + hair	0·49	nd	nd	tr	tr	nd	0·43	tr	0·17	tr	0·20
Gall-bladder (+contents)	7·11	8·65	7·19	nd	nil	12·8	52·6	nil	8·10	nil	nd
Urine: Day 1	nd	32	nd	155	70	nd	nd	292	174	344	205
2	..	(1hr.)	..	..	27	..	..	..	..	64	6
3	..	..	..	..	29	..	..	..	..	25	36
4	..	..	..	..	24	..	..	..	..	22	54
5	..	..	..	..	14	..	..	..	..	14	50
6	..	..	..	..	17	..	..	..	..	nil	50
7	..	..	..	..	20	..	..	..	..	8	..
Fœces: Day 1	nd	nd	nd	3	12	nd	nd	31	4	85	no fæces
2	..	..	..	..	9	..	..	..	..	106	,,
3	..	..	..	..	nil	..	..	..	..	47	,,
4	..	..	..	..	9	..	..	..	..	47	,,
5	..	..	..	..	8	..	..	..	..	nd	,,
6	..	..	..	..	nil	..	..	..	..	9	,,
7	..	..	..	..	14	..	..	..	..	nd	..
Total As in body, μg./mg. injected	1386	816	940	188	596	578	688	tr	149	17	101
Total As accounted for, μg./mg. injected	..	..	..	346	849	..	..	323	327	789	502

TABLE I—*Continued*.

Expt. no.	4.	2.	6.	1.	9.	18.	17.	19.	20.	21.	22
Arsenical	Phenylarsonic acid.							m-Amino-p-hydroxy-phenylarsonic acid.			
Time killed, hrs.	1	1	24	24	24	168	168	1	1	24	24
Dose, mg. As/kg.	1·11	5·56	1·11	5·56	5·56	1·11	5·56	4·62	14·2	16 1	16 1
Wt. of rabbit, kg.	2·50	2·80	2·40	2·45	2·10	2·40	2·20	2 05	2 6	2 5	1·7
Sex	F.	M.	M.	F.	M.	M.	M.	F.	F.	M	M
Lung	0·96	0·70	0·53	0·58	0·12	nil	nil	0·17	0 32	tr	Tissues not analysed
Liver	1·88	0·95	0·63	0·50	0·55	0 27	nil	0·45	0 32	0 84	
Kidney	3·53	3·69	0·43	0·55	0·21	nil	nil	4 08	4·50	0 14	
Blood cells	0·37	0·65	nil	0 04	0·05	nil	nil	0·43	0 50	nil	
Plasma	0·78	0·95	nil	0·02	nil	nil	nil	0·42	0 37	nil	
Small intestine	0·28	0·23	tr	0 03	0 07	nil	nil	0 09	0 13	0 10	
Muscle (vol.)	0·16	0·09	0·24	0·05	nil	tr	nil	0 14	0 06	nil	
Brain	nil	nil	tr	nil	nil	nil	nil	nil	nil	nil	
Heart	tr	0 44	0·99	0·17	nil	nil	nil	tr	0·18	0 03	
Bone (rib) + marrow	nd	nd	nil	nd	nil	0 22	nil	0 16	0 15	0 01	
Bone marrow	nil	0·52	tr	0·42	nil	nil	nd	nil	tr	nil	
Spleen	tr	nil	nil	0·17	nil	nil	nil	nil	0 22	0 11	
Skin + hair	nd	nd	0·42	nd	tr	0·09	nil	nd	0·54	nil	
Gall-bladder (+con-tents)	nil	tr	tr	nil	nil	nd	nil	nil	0 20	tr	
Urine: Day 1	nd	nd	777	nd	698	827	677	nd	nd	690	840
2						22	3				
3						12	2				
4						8	1				
5						4	1				
6						5	2				
7						2	1				
Fæces. Day 1	nd	nd	2	nd	6	7	25	nd	nd	1	5
2						6	nil				
3						5	nil				
4						3	nil				
5						1	0·1				
6						2	0 1				
7						2	nil				
Total As in body, μg./mg. injected	268	209	198	57	36	36	nil	171	200	51	..
Total As accounted for, μg./mg. injected	..	..	977	..	740	942	712	..	..	742	..

weight, before entry in the table. With even distribution of arsenic throughout the body, and without any excretion, the figure in the table for each tissue would be 1·0. "Trace" indicates that the amount of arsenic present, while detectable, was so small that for that particular sample the analytical results had no quantitative value. In no case in which "trace" appears in the table was there more than 4 μg. As in the weight of tissue taken for analysis, excluding As in reagents. The weights of the samples analysed were governed largely by the sizes of the organs and ranged from about 1 to 20 g.

Results for urine and fæces are given in the table as μg. As excreted

for each mg. As injected into the whole animal. Complete excretion would thus be represented by the figure 1000.

The use of this rather abstract notation to express the experimental results is felt to be justified since it facilitates comparison between different experiments by eliminating the variables body-weight and dosage, and since the results thus expressed lend themselves to further calculation. The figures shown in Table I for total arsenic remaining in the body at the time of sacrifice were calculated by means of data

TABLE II.—FATE OF THE ARSENIC AFTER INJECTION OF PHENYLARSENOXIDE.
(Expt. No. 16. Rabbit killed 168 hours after injection of 0·45 mg. As/kg.)

	(A) μg. As/gm. for dose of 1 mg. As/kg.	(B) Wt. of tissue, gm./kg. body-wt.	(A × B) μg. As per mg. injected.
Lung	tr	6	tr
Liver	1·47	34	50
Kidney	1·67	7	12
Blood cells	tr	22	tr
Plasma	tr	44	tr
Muscle (vol.)	0·76	486	368
Heart	nil	3	nil
Bone	tr	75	tr
Skin + hair	tr	125	tr
Other tissues	0·84	198	166
Total As in body			596
Total As in urine			201
Total As in fæces			52
Total As accounted for			849

given by Levine *et al.* [1941] for the average weights of the organs and tissues of the rabbit. Table II shows the calculation in detail for one experiment. It will be seen that multiplying the arsenic concentration (as expressed in Table I) in any one tissue by the weight of the tissue in g./kg. body-weight gives the arsenic in the total weight of the tissue as μg./mg. injected, *i.e.* in the same terms as the excretion figures. It was assumed in these calculations that the cells accounted for one-third of the total weight of blood and that those tissues not listed had the same average arsenic content as small intestine, for which no separate details are given by Levine *et al.* The weight of "other tissues" (including small intestine) was arrived at by difference. It should be noted that in working out the fraction of the body-weight represented by each tissue, the weight of blood was included in the total. This was not done by Levine *et al.*

DISCUSSION.

With none of the arsenicals studied does variation in dosage appear to have affected the relative distribution of arsenic in the body to any great extent. Phenylarsenoxide was in contrast to the other arsenicals in that a very high concentration of arsenic was found in the lungs one
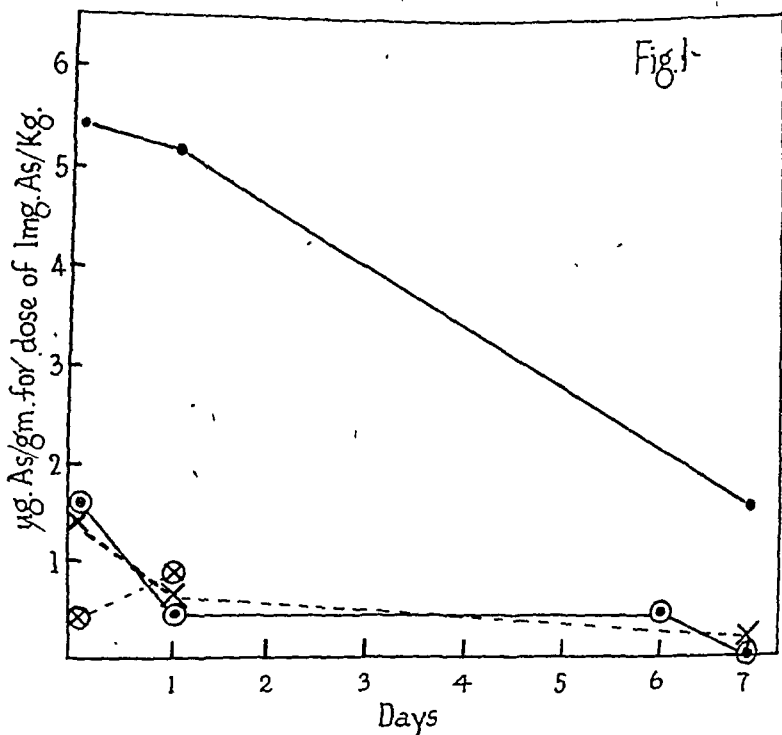
Fig. 1.—Average arsenic content of lung at various periods.

●——●, phenylarsenoxide.
⊙——⊙, mapharsen.
×——×, phenylarsonic acid.
⊗——⊗, m-amino-p-hydroxyphenylarsonic acid.

hour after injection. Twenty-four hours after administration of phenylarsenoxide, however, the arsenic in the lungs was only just detectable. In comparison with other organs the liver and kidney took up relatively large amounts of all four compounds per unit weight of tissue. The proportion of the dose of As taken up by these organs was particularly high after the injection of phenylarsenoxide. An appreciable amount of arsenic was still present one week after the injection of this compound (fig. 1). Little or no arsenic was present in the blood plasma or cells twenty-four hours after injection of any of the arsenicals. It is of interest to note the immediate fixation of arsenic by the blood cells

which occurred after injecting phenylarsenoxide, but which was not seen in the case of either arsonic acids. Intestine and heart had, on the whole, higher arsenic contents than voluntary muscle. Since it represents a large fraction of the total body-weight (Table II), voluntary muscle may contain a great part of the arsenic in the body, in spite of the relatively low arsenic concentrations usually found in this tissue.
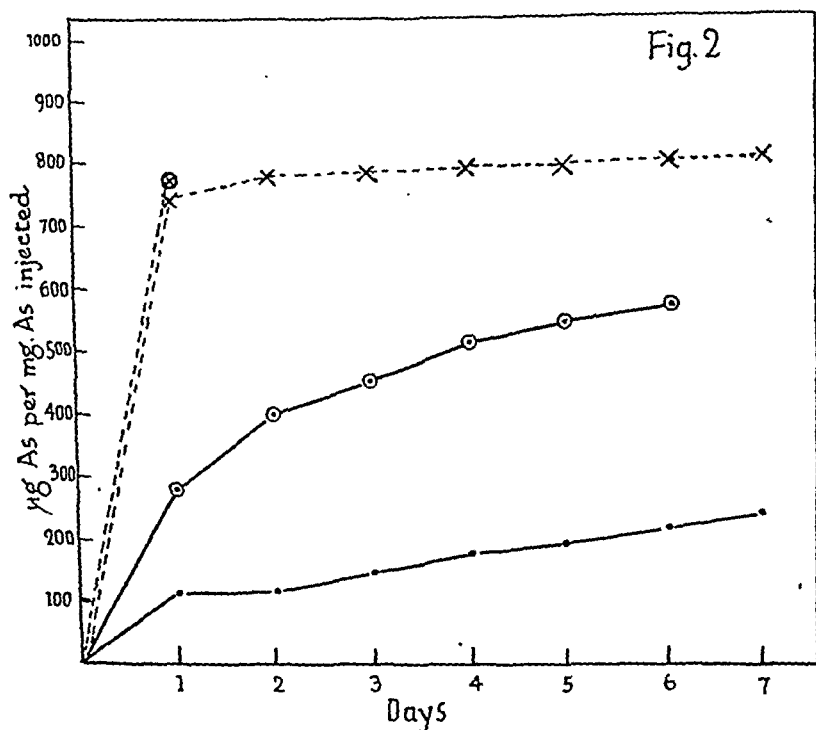
FIG. 2.—Cumulative daily excretion of arsenic in urine and fæces combined
(averaged results).

●——●, phenylarsenoxide.
⊙——⊙, mapharsen.
×——×, phenylarsonic acid.
⊗——⊗, m-amino-p-hydroxyphenylarsonic acid.

The intestinal tract is probably also important in this respect. In these two tissues, a higher concentration of arsenic in proportion to the dosage was present following injection of phenylarsenoxide than after either of the arsonic acids. Mapharsen occupied an intermediate position in this respect. It is of interest to note that in no experiment was arsenic present in the brain in measurable amounts.

With both arsenoxides, in contrast to the arsonic acids, the figures for arsenic in the gall-bladder and its contents suggest that there was excretion of arsenic in the bile, but from the amounts of arsenic found

in the fæces reabsorption may have occurred. The total amount of arsenic excreted with the fæces was much less than in the urine in all experiments, the difference between the two modes of excretion being less marked with the arsenoxides than with the arsonic acids. Fig. 2 shows the course of the arsenic excretion in urine and fæces combined. It can be seen that excretion was quicker and more complete with the two arsonic acids than with mapharsen, and in turn with mapharsen than with phenylarsenoxide. In one experiment with mapharsen (No. 12) the animal was in such poor condition that it had to be killed six days after injection, and its condition may have affected the excretion. Only one experiment of a week's duration was carried out with phenylarsenoxide. However, the findings for the excretion of this compound and of mapharsen agree with the results obtained by Hogan and Eagle [1944].

Although too much weight should not be attached to the calculated figures for arsenic remaining in the body, these give the same picture for the relative rates of clearance of the four arsenicals as that obtained from the excretion results. No explanation can be offered for the poor recoveries of arsenic in experiments 8, 13, and 14.

Figures quoted in the literature for the toxicities of the four arsenicals dealt with in this report on intravenous injection into rabbits are shown in Table III. It seems that the differences in toxicity, including that between the two arsenoxides, can be partly explained by the differences in the rates of excretion (fig. 2) and in the amounts of arsenic fixed by the body cells. Thus the lower toxicity and correspondingly greater chemotherapeutic index [Eagle et al., 1940] of mapharsen compared with phenylarsenoxide may be due to a direct or indirect effect of the substituents in the benzene ring in hastening clearance. However, the excretion of phenylarsonic acid is as rapid as that of m-amino-p-hydroxyphenylarsonic acid, suggesting that the valency of the arsenic is of more direct importance than substitution in the phenyl group in this particular.

Hogan and Eagle [1944] also concluded that, in uncomplicated cases, the toxicity of an arsenical is related to the rate of excretion and that both are dependent on the extent to which arsenic is bound by the body tissues. They found that fixation of arsenic by red blood cells in vitro provided a model for fixation by tissues in vivo. Their figures for tissue arsenic contents after injection of rabbits with phenylarsenoxide, phenylarsonic acid, or mapharsen are, where comparable, in agreement with our own in most respects. This is also true for results obtained by other workers who studied the distribution of mapharsen in the body [Magnuson and Raulston, 1941; Wright et al., 1937], except in the case of bone, in which very high arsenic contents were found in Magnuson and Raulston's experiments with dogs.

Results obtained by I. D. E. Storey [unpublished work] in a study

TABLE III.—TOXICITIES OF ARSENICALS FOR RABBITS ON INTRAVENOUS INJECTION.

Compound.	Formula of solid used.	Lethal dose, mg./kg.	Reference.
Phenylarsenoxide	C_6H_5AsO	0·79	Eagle et al. [1940].
m-Amino-p-hydroxy-phenylarsenoxide	$3-NH_3Cl-4-OH-C_6H_3AsO$, $\frac{1}{2}C_2H_5OH$	13·3	Eagle et al. [1940].
Phenylarsonic acid	$C_6H_5AsO_3HNa$	70	Young and Loeven-hart [1924].
	$C_6H_5AsO_3H_2$	16	Hogan and Eagle [1944].
m-Amino-p-hydroxy-phenylarsonic acid *	$3-NH_2-4-OH-C_6H_3AsO_2Na_2$	100	Young and Loeven-hart [1924].

* From a figure given for the toxicity of this compound in mice by Hogan and Eagle [1944], who find that mouse toxicities generally parallel rabbit toxicities, it seems possible that the median lethal dose of this compound on intravenous injection into rabbits is nearer 500 mg./kg.

of the fate of inorganic arsenite and arsenate in the body confirmed the view of Voegtlin and Thompson [1922] that the difference in the toxicities of these two compounds for the whole animal can be explained from the different rates at which the arsenic is cleared from the body. In addition to determining total arsenic, Storey carried out separate estimations of arsenite and arsenate in the urine by the method of Crawford and Storey [1944], and came to the conclusion that the rate of clearance of arsenite was governed by the rate of its oxidation in the body to arsenate. It seems possible that the more rapid clearance and the lower toxicity of mapharsen as compared with phenylarsenoxide are both due, in part at least, to readier conversion in the body to the corresponding arsonic acid. The toxicity and the rate of excretion of an organic trivalent oxide of arsenic may be determined to some degree by the rate of its oxidation in the body to the pentavalent arsenical.

It is unfortunate that the amounts of arsenic in blood, brain, and cerebrospinal fluid after administration of organic arsenicals are usually too small for accurate measurement, in spite of the very sensitive quantitative chemical methods now available for arsenic determination in biological material [Chaney and Magnuson, 1940; Cassil and Wichmann, 1939; Levvy, 1943]. The use of radio-isotopes of arsenic should yield valuable information regarding these very important fractions of the arsenic administered. So far, work on the distribution and excretion of radio-arsenic has dealt only with arsenite and arsenate [Born and Timofeeff, 1941; Du Pont et al., 1942; Hunter et al., 1942].

SUMMARY.

1. The effect of changing the valency of the arsenic and of intro-
ducing substituents into the benzene ring on the distribution in the
body and excretion of an aromatic arsenical has been studied, using
phenylarsenoxide, phenylarsonic acid, *m*-amino-*p*-hydroxyphenylarsen-
oxide (mapharsen), and *m*-amino-*p*-hydroxyphenylarsonic acid, injected
intravenously into rabbits.

2. A high arsenic content was found in liver and kidney in all cases,
and also in lung in the case of phenylarsenoxide.

3. The ratio between the total amounts of arsenic excreted in the
urine and in the fæces varied widely with the different compounds, but
the urinary output predominated in all experiments. The arsonic
acids were cleared from the body more rapidly than the corresponding
arsenoxides. Of the latter, unsubstituted phenylarsenoxide was the
more slowly excreted.

4. The slow rate of clearance of phenylarsenoxide may explain its
high toxicity as compared with the other arsenicals studied. It is
thought that the slow excretion and high toxicity of phenylarsenoxide,
as compared with mapharsen, may be due to slower oxidation to the
corresponding arsonic acid.

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SUMMARY.

1. The effect of changing the valency of the arsenic and of intro-ducing substituents into the benzene ring on the distribution in the body and excretion of an aromatic arsenical has been studied, using phenylarsenoxide, phenylarsonic acid, m-amino-p-hydroxyphenylarsen-oxide (mapharsen), and m-amino-p-hydroxyphenylarsonic acid, injected intravenously into rabbits.

2. A high arsenic content was found in liver and kidney in all cases, and also in lung in the case of phenylarsenoxide.

3. The ratio between the total amounts of arsenic excreted in the urine and in the fæces varied widely with the different compounds, but the urinary output predominated in all experiments. The arsonic acids were cleared from the body more rapidly than the corresponding arsenoxides. Of the latter, unsubstituted phenylarsenoxide was the more slowly excreted.

4. The slow rate of clearance of phenylarsenoxide may explain its high toxicity as compared with the other arsenicals studied. It is thought that the slow excretion and high toxicity of phenylarsenoxide, as compared with mapharsen, may be due to slower oxidation to the corresponding arsonic acid.

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THE EFFECT OF SECTION OF THE SUPRAOPTICO-HYPOPHYSEAL TRACTS ON THE INHIBITION OF WATER-DIURESIS BY EMOTIONAL STRESS. By W. J. O'CONNOR (Beit Memorial Research Fellow). From the Pharmacology Laboratory, Cambridge.

RYDIN and VERNEY [1938] and O'Connor and Verney [1942, 1945] investigated the inhibition of urine flow which occurs in the dog following emotional stress induced during water-diuresis. Two types of inhibition were distinguished by O'Connor and Verney [1945]—a *rapid* type due to vaso-constriction in the kidney during the emotion, and a *slow*·type due to the release of antidiuretic substance from the neurohypophysis and almost completely abolished by removal of the posterior lobe of the pituitary [O'Connor and Verney, 1942]. After denervation of the kidneys and suprarenal glands, the slow type occurred consistently in all tests and in all animals [O'Connor and Verney, 1945], and so the presence of a *slow* inhibition of water-diuresis in response to emotional stress in animals previously submitted to denervation of the kidneys and suprarenals forms a test of the functional activity of the neurohypophysis.

Fisher, Ingram, and Ranson [1938] produced permanent diabetes insipidus in cats and monkeys by lesions in the hypothalamus so placed as to destroy the supraoptic nuclei or to interrupt the supraopticohypophyseal tracts, and evidence was presented which strongly supported the explanation that the polyuria was due to lack of antidiuretic substance in consequence of atrophy of the neurohypophysis following the tract section. In this paper the function of the neurohypophysis after section of the supraoptico-hypophyseal tracts in the dog will be tested by experiments designed to demonstrate that, in dogs rendered polyuric by tract section, emotional stress causes no release of antidiuretic substance as shown by the absence of the *slow* type of inhibition of water-diuresis, the kidneys and suprarenals having been previously denervated.

METHODS.

The experimental procedures in these experiments were those described by O'Connor and Verney [1942]. Four bitches were used, all being fully-grown animals between 8·5 and 12 kg. in weight. Before

was quite as great as that which caused the large inhibition of fig. 1, but in the tests after tract section illustrated in fig. 2, only small inhibitions
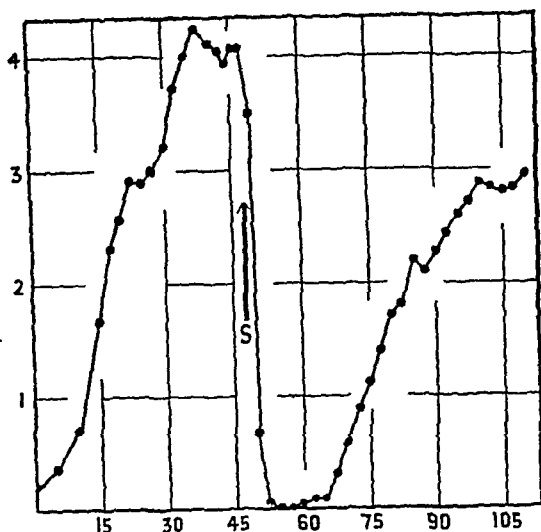
FIG. 1.—Inhibition of water-diuresis by emotional stress in a bitch before section of the supraoptico-hypophyseal tracts but after denervation of kidneys and supra-renals; "Tanner I", weight 8·5 kg. The test dose of 300 c.c. of water was given at zero time, and times in minutes after the administration of the water are plotted as abscissæ. Ordinates show the rate of urine secretion in c.c. per minute during the period preceding the plotted point. All graphs in this paper are plotted in this way. At S the dog was excited by faradic stimulation applied to the flanks for one minute.
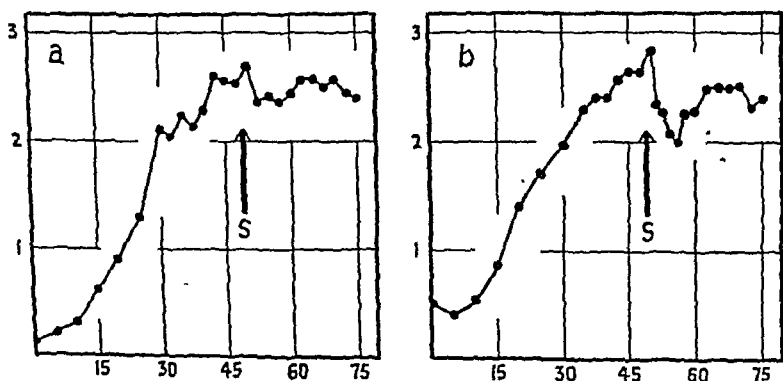
FIG. 2.—The effect of the same emotional stimulus on the same animal as in fig. 1, but 35 (a) and 70 (b) days after section of the supraoptico-hypophyseal tracts. Ordinates and abscissæ as in fig. 1.

of the diuresis followed. In all, 44 tests of the effect of emotional stress were made on the four dogs after tract section; 29 resulted in inhibitions

the observations were begun each animal was submitted to the operation described in the previous paper in which the kidneys were denervated, the splanchnic nerves cut, and the second, third, and fourth lumbar ganglia excised with the sympathetic chain between them; it is this operation which has been referred to above as "denervation of the kidneys and suprarenals."

To section the supraoptico-hypophyseal tracts, the pituitary region was exposed through the mouth by the diasphenoid route, the method being similar to that used for the removal of the posterior lobe [Pickford, 1939; O'Connor and Verney, 1942]. For the present purpose the incision in the bone was made further forward so that, when the inner table had been removed, the anterior lobe of the pituitary was visible in the posterior third of the hole and the optic chiasma at the anterior edge. The dura was then incised to expose the tuber cinereum; with a tenetome a stab wound was made between the optic chiasma and the pituitary to a depth of about 0·4 cm., and the superficial extent of this wound carried on each side as far laterally and caudalwards as possible. In one animal diathermy with a narrow blade was used to make the lesion. As in the previous paper [O'Connor and Verney, 1942], at the conclusion of the observations on each animal, the brain was fixed *in situ* and serial sections of the pituitary and hypothalamic regions cut, so that the precise nature of the lesion could be determined, as well as the consequent changes in the pituitary and hypothalamus.

RESULTS.

The Effect of Tract Section on the Inhibition of Water-Diuresis by Emotional Stress.

In each animal after denervation of the kidneys and suprarenals, a large inhibition of water-diuresis was obtained at each test of the effect of emotional stress, the inhibition being of the slow type, but, after section of the supraoptico-hypophyseal tracts, emotional stress induced during water-diuresis caused no or only a very small inhibition of the urine flow. Figs. 1 and 2 illustrate the experiment on one dog. Fig. 1 shows the diuresis in response to 300 c.c. of water after denervation of the kidneys and suprarenals but before any operation on the pituitary or hypothalamus. Forty-seven minutes after the administration of the water the rate of urine flow had risen to 4 c.c. per minute; faradic stimulation was then applied to the flanks for 1 minute causing mild resentment. Seven minutes later the urine flow had ceased, at 14 minutes after the stimulus the rate of flow began to increase again, and by 50 minutes from the time of stimulation the rate of flow had recovered to about 3 c.c. per minute. Fig. 2, *a* and *b*, shows the result on the same animal using the same stimulus but 35 and 70 days after section of the supraoptico-hypophyseal tracts. In each case the emotional disturbance

hibitions by emotional stress is more prolonged than that due to 5 mU [1] but shorter than that due to 10 mU. In all, 5 tests were made on this animal of the inhibition by a stimulus of this strength and duration, and each was assayed as the equivalent of between 5 and 10 mU of pituitary antidiuretic substance. Usually the inhibition was nearer to that of 5 mU than to that of 10, and so it is estimated that the average release of antidiuretic substance in response to the standard stimulus in this dog was 6·5 mU. After tract section a similar assay was made of the

FIG. 4.—The estimation of the amount of antidiuretic substance liberated in response to the same emotional stimulus with the same dog as in fig. 3, but after section of the supraoptico-hypophyseal tracts. (a) The inhibitions resulting from the intravenous injection of 0·1, 0·3, and 0·5 mU of post-pituitary extract. (b) Two inhibitions by one minute of faradic stimulation; S_1 22 and S_2 44 days after tract section. Ordinates and abscissæ as in fig. 1.

residual inhibition by the same faradic stimulus, and fig. 4 shows this part of the experiment on "Tanner II". Fig. 4, a, presents the inhibitions which resulted from the intravenous injection of 0·1, 0·3, and 0·5 mU of posterior lobe extract; fig. 4, b, two inhibitions which resulted from a faradic stimulation of the same strength and duration as that used before operation. In the one case the inhibition is equivalent to that due to 0·1 mU (S_1) and in the other 0·3 (S_2). In all, 9 such tests of the effect of emotional stress were made after tract section on this animal; 2 of the responses were assayed as equivalent to 0·3 mU, 3 as 0·1 mU, and in 4 the inhibition was less than that due to 0·1 mU of antidiuretic

[1] The extract used was that marketed by Messrs. Burroughs Wellcome under the name "Infundin", and standardized to contain 10 international oxytocic units per c.c. One milli-unit (mU) signifies the antidiuretic activity of 10^{-4} c.c. of the extract.

clearly smaller than that of fig. 2, *b*, there were 13 inhibitions of approximately the same size and in only 2 tests was there an inhibition larger than that of fig. 2, *b*.   Thus, it is concluded that after section of the supraoptico-hypophyseal tracts, the *slow* inhibition of water-diuresis by emotional stress is almost, but not completely, absent and it follows that
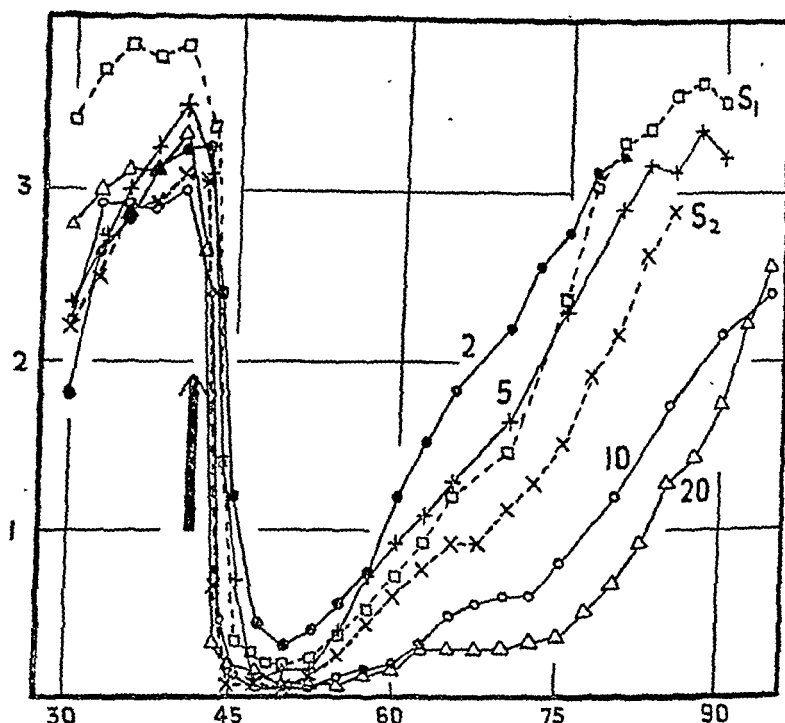
Fig. 3.—The estimation of the amount of antidiuretic substance liberated in response to one minute of faradic stimulation before tract section; "Tanner II", weight 10 kg.; kidneys and suprarenals denervated. Dose of water (300 c.c.) was given at zero time; ordinates and abscissæ are as in fig. 1 but the early parts of the curves are not shown. In each case the stimulus or the injection was given at the arrow. Curves 2, 5, 10, 20 are inhibitions produced by 2, 5, 10, 20 mU post-pituitary extract; S_1 and S_2 inhibitions by one minute of faradic stimulation.

only a greatly diminished amount of antidiuretic substance is discharged under such circumstances.

The residual inhibition by emotional stress after tract section was quantitatively estimated by the method described in detail by O'Connor and Verney [1942]; figs. 3 and 4 *illustrate the experiment on one animal.* In fig. 3 comparison is made between two inhibitions produced by 1 minute of faradic stimulation after denervation of the kidneys and suprarenals but before tract section, and a series of inhibitions due to the intravenous injection of post-pituitary extract; each of the in-

·hibitions by emotional stress is more prolonged than that due to 5 mU [1] but shorter than that due to 10 mU. In all, 5 tests were made on this animal of the inhibition by a stimulus of this strength and duration, and each was assayed as the equivalent of between 5 and 10 mU of pituitary antidiuretic substance. Usually the inhibition was nearer to that of 5 mU than to that of 10, and so it is estimated that the average release of antidiuretic substance in response to the standard stimulus in this dog was 6·5 mU. After tract section a similar assay was made of the
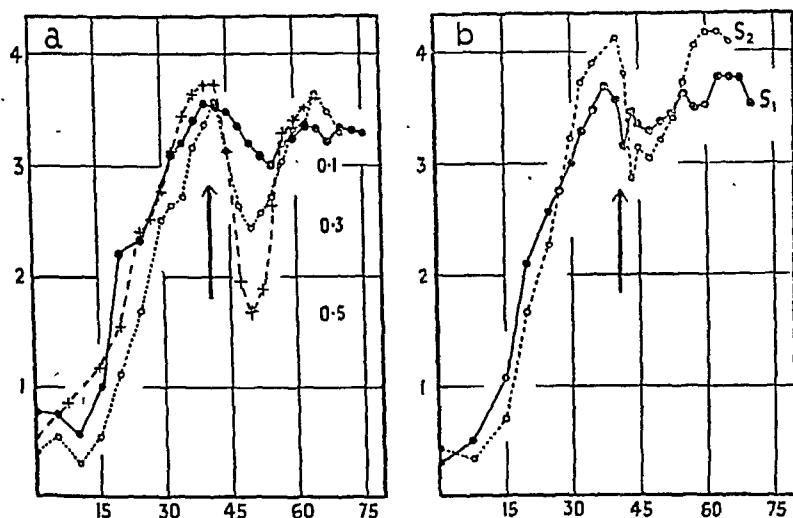

Fig. 4.—The estimation of the amount of antidiuretic substance liberated in response to the same emotional stimulus with the same dog as in fig. 3, but after section of the supraoptico-hypophyseal tracts. (a) The inhibitions resulting from the intravenous injection of 0·1, 0·3, and 0·5 mU of post-pituitary extract. (b) Two inhibitions by one minute of faradic stimulation; S_1 22 and S_2 44 days after tract section. Ordinates and abscissæ as in fig. 1.

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substance.   The average release was 0·15 mU of antidiuretic activity, as the result of the same stimulus which before operation caused the liberation of 6·5 mU.   It is therefore estimated that approximately 2 per cent. of the neurohypophysis was intact after tract section in this animal.   Similar assays with the other three dogs gave 2, 7, and less than 6 per cent. of normal as the size of the residual inhibition in each case.

The Anatomical Changes in Pituitary and Hypothalamus.

In introducing the description of the lesions produced by the operation of tract section, it is necessary first to discuss briefly the normal anatomy of the region.   In the dog the pituitary stalk itself is short, but there is a long ventral wall where the floor of the third ventricle becomes evaginated and its sides folded together to form the stalk. Fig. 5, a. is the midline sagittal section of this region in a normal dog, reconstructed by measurement of the frontal sections into which the block had been cut, and shows the long ventral and the short dorsal wall of the stalk; fig. 6, a and b are two frontal sections to show the evagination of the floor of the ventricle and the folding of the walls to form the stalk.   Sections stained by Bodian's [1936] colloidal silver method reveal dense bundles of nerve fibres of the supraoptico-hypophyseal tracts running back in the floor of the ventricle into the ventral wall of the stalk and so into the pars nervosa, and further fibres can be seen entering at the sides of the ventral wall in the directions shown by the dotted lines in fig. 6, a and b.   With these relationships in mind we may proceed to the description of the lesion produced in one dog ("Tanner II") in which the findings are typical of the animals submitted to tract section.

Fig. 5, b is the reconstructed midline section of this dog, and the scar tissue (cross-hatched in the figures) shows the site of the main stab wound.   The knife entered the tuber cinereum just anterior to the anterior lobe of the pituitary and about 1 mm. behind the optic chiasma. Thence the cut passed upwards and backwards across the third ventricle, its course being traced by the damage to the lateral walls of the ventricle causing them to adhere in some places, and by areas of scarring at the posterior lip of the stalk of the pituitary (X in fig. 5, b) and in the posterior wall of the ventricle (at Y).   The lateral extent of the scar is seen in the frontal sections of fig. 6, c–h.   In fig. 6, e, and in sections 0·1 and 0·2 mm. anterior to it (not reproduced), the scar extended completely across the evagination of the ventricle with the exception of a narrow strip on the right side (Z in fig. 6, e) where a scanty network of nerve fibres was found throughout the sections adjacent to that of fig. 6, e.   Behind this level (fig. 6, f) the floor of the evagination was not directly damaged, but the lateral wall has been severed on each side by lesions extending back beyond the posterior wall of the stalk (fig. 6, g).   Thus the lesion was

V-shaped and so placed as to interrupt the fibres of the supraoptico-hypophyseal tracts as they enter the anterior and lateral aspects of the
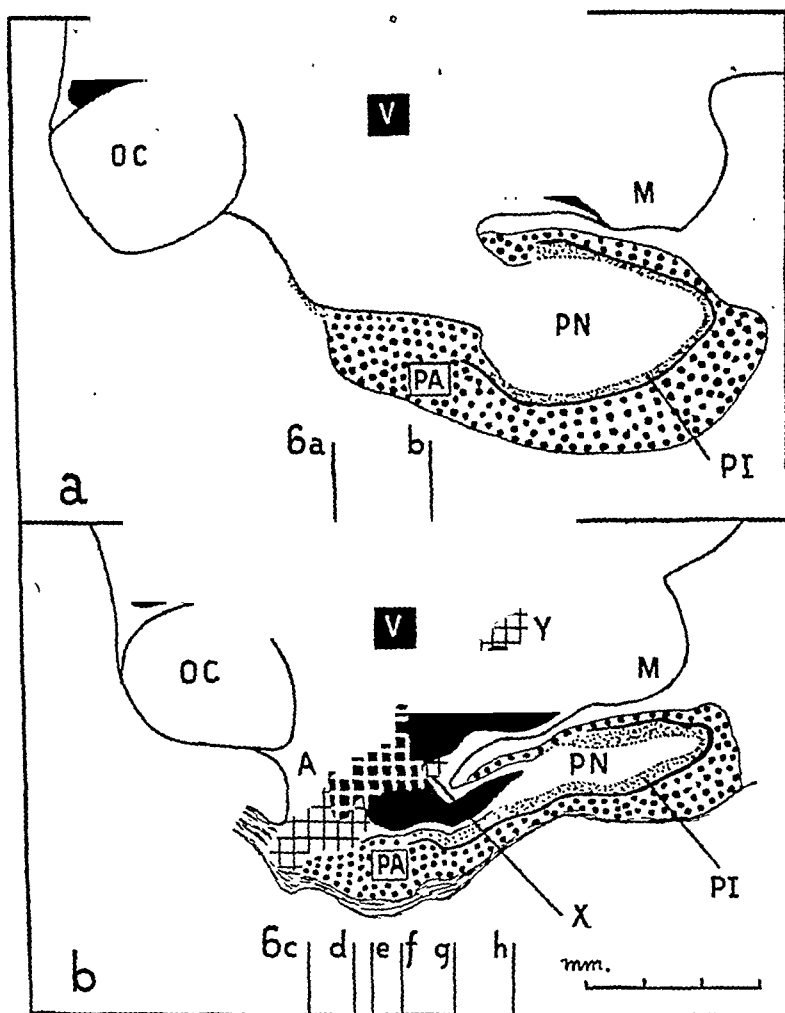
Fig. 5.—(a) Midline sagittal section of the hypothalamic and hypophyseal regions of a normal bitch; weight 10 kg. (b) Similar section from a bitch after section of the supraoptico-hypophyseal tracts; "Tanner II", weight 10 kg. In this and in fig. 6 scar tissue in the nervous structures is indicated by cross-hatching. OC, optic chiasma; OT, optic tract; M, mamillary bodies; F, column of the fornix; PA, pars anterior; PN, pars nervosa; PI, pars intermedia; V, third ventricle. Other lettering is referred to in the text. The lines 6a, b, etc., indicate the planes of the frontal sections of fig. 6. Enlargement × 8.

stalk, and the interruption of the fibres is shown by the sections stained with silver. Anterior to the lesion, the floor of the third ventricle (A in figs. 5, b, 6, c and d) contains a network of nerve fibres, and fibres
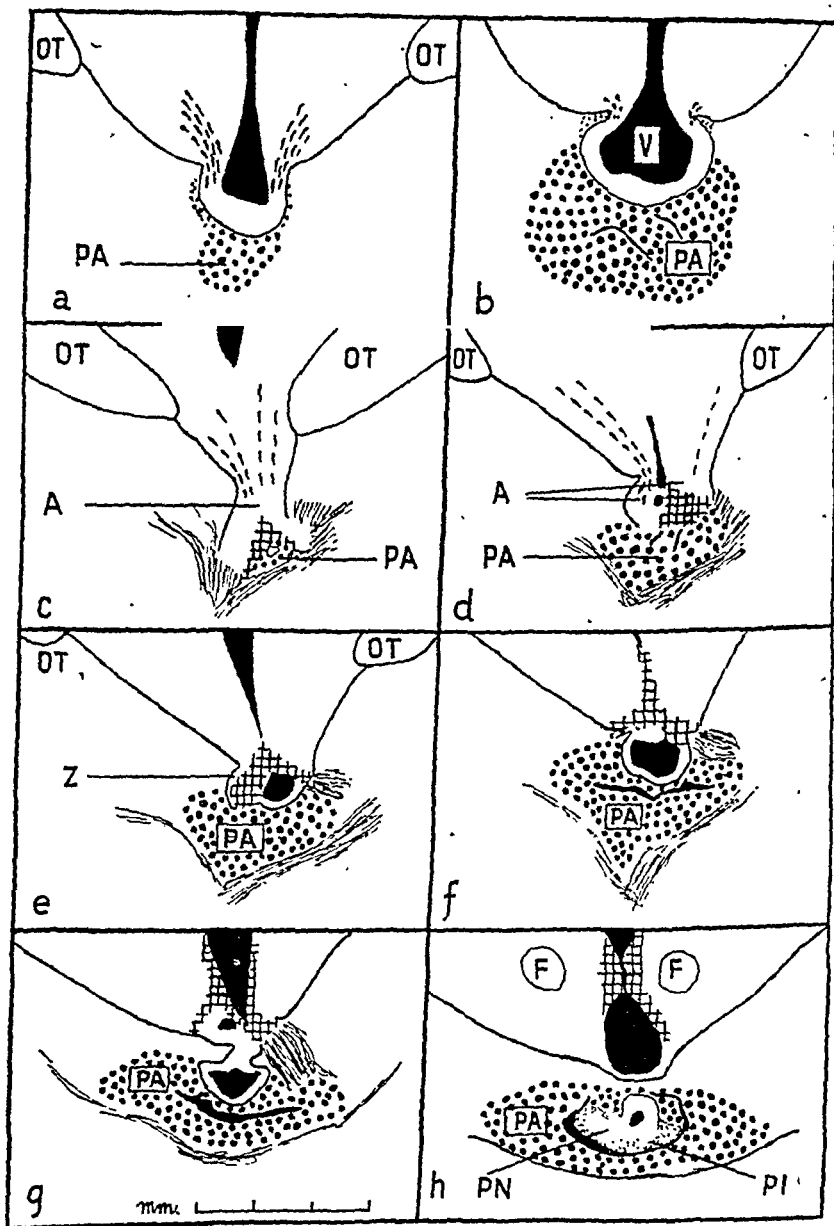
FIG. 6.—(a) and (b) Frontal sections through the hypothalamus and pituitary stalk of the normal dog in the planes indicated in fig. 5, a. (c)–(h) Frontal sections of the region in "Tanner II" in the planes indicated by the lines in fig. 5, b. Lettering as in fig. 5. Enlargement ×8.

can be seen streaming in as shown by the dotted lines of fig. 6, c and d. There is, however, no mass of longitudinally running fibres as is present in the normal dog.   Behind the lesion, the walls of the evagination are reduced in thickness from about 0·4 mm. in the normal animal to 0·15 mm., and the bundles of nerve fibres which pass along the stalk are completely absent, only an occasional nerve fibre being seen in the silver sections.   Similarly the pars nervosa is greatly shrunken (compare fig. 5, a and b) and there remain but few nerve fibres of the bundles and network present in the normal gland.   In agreement with Fisher, Ingram, and Ranson [1938], there is hypercellularity of the shrunken stalk and pars nervosa, and in the hypothalamus there is obvious loss of cells from the supraoptic nuclei.   The pars anterior and pars intermedia remain normal.   The diagrams also show other damage in the hypothalamus confined to the walls of the ventricle, with obliteration of the cavity in some places and enlargement at others (fig. 6, e–h), while the sagittal sections of fig. 5 show the distortion of the ventricle produced by the retraction of the scar adherent to the overlying dura.

The examination of two of the remaining animals revealed pictures in all respects similar, the essential lesion being the section of the anterior and lateral walls of the ventral prolongation of the stalk.   In the other dog ("Tanner I"), the experiment on which is illustrated in figs. 1 and 2, the initial stab wound was in the same situation as that illustrated in fig. 5, b, but did not extend to a depth greater than necessary to sever the ventral wall of the evagination and there was no extension backwards or to either side.   Nerve fibres were found entering the lateral sides of the stalk in considerable numbers and could be traced back along the stalk to the pars nervosa; obviously the supraoptic-hypophyseal tracts were not completely interrupted in this case, although the great mass of fibres in the anterior wall of the stalk was absent and the usual shrinkage and hypercellularity of the pars nervosa were found.

Polyuria after Tract Section.

From time to time before and after tract section, the animals were kept in metabolism cages to enable a record of the daily urine volume to be obtained; during a period of urine collection the diet was always 8 oz. of dog biscuits per day with water *ad libitum*.   Fig. 7 shows the polyuria which resulted from tract section in one of the animals.   The onset of the polyuria was in three phases as described by Fisher, Ingram, and Ranson [1938] for cats and monkeys; thus in fig. 7 there is a "temporary polyuria" until the 7th day after tract section, then there is a "normal interphase" from the 7th–10th days, and thereafter the "permanent polyuria".   Fig. 7 is typical of the results in all four animals, the three phases being clearly present in each experiment.

In three of the animals an increase in the daily urine volume persisted

throughout the four months that the animals were kept, although in two of these ("Tanner II"; "Span", fig. 7) the degree of polyuria decreased during this time. In the third animal ("Hobo") a high degree of polyuria was maintained throughout. The remaining animal ("Tanner I") showed a definite temporary polyuria and interphase, but in the permanent phase excreted only 1·5 times the preoperative volume of urine. It was in this animal that the post-mortem revealed
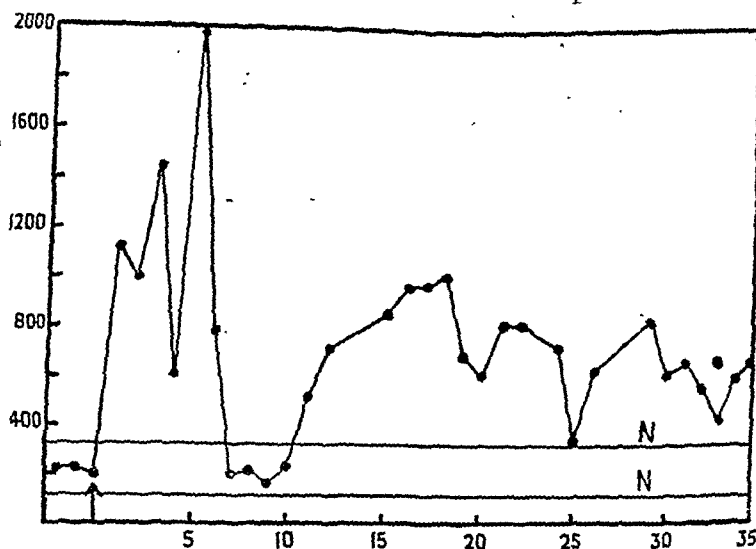
FIG. 7.—The daily urine volume after section of the supraoptico-hypophyseal tracts; "Span", weight 10 kg., kidneys and suprarenals denervated. The tract section was at the arrow, and days after operation are plotted as abscissæ. The daily urine volume in c.c. is plotted as ordinates. Urine volume was measured at 9.30 a.m. and each plotted point shows the volume during the preceding 24 hours. The lines NN indicate the range of the daily urine volume in this animal before operation.

fibres of the supraoptico-hypophyseal tracts, not sectioned at the operation, reaching the stalk and posterior lobe.

In three of the animals tests of the inhibition of water-diuresis by emotional stress were made on the days immediately following the tract section, during the period of the temporary polyuria. In each case the animal was apparently quite well and responded to the stimulation with the usual mild resentment, but in all tests only a small inhibition of water-diuresis occurred; it was no larger than the inhibitions subsequently observed in response to the same stimulus during the permanent phase of the polyuria.

DISCUSSION.

Section of the supraoptico-hypophyseal tracts, carried out in dogs by the method described in this paper, has results entirely similar to

those described by Fisher, Ingram, and Ranson [1938] using cats and monkeys; polyuria supervenes with the typical three stages in its onset; there is a loss of cells from the supraoptic nuclei; and the pars nervosa and stalk of the pituitary shrink, contain more cells than normal, and lose their bundles and network of nerve fibres. Fisher and Ingram [1936] have described experiments which show that extracts of the shrunken neurohypophysis of diabetic cats contain less than 8 per cent. of the pressor, oxytocic, and antidiuretic activity found in the normal neurohypophysis; Hickey, Hare, and Hare [1941] similarly found no antidiuretic activity in extracts of the posterior lobes of rats after section of the pituitary stalk. We can now add the additional fact that after tract section there is only a small inhibition of water-diuresis by emotional stress, corresponding to the release of less than 7 per cent. of the amount of antidiuretic substance liberated into the blood-stream in response to the same stimulus before operation. Thus, this new fact agrees with other evidence that the interruption of the supraoptico-hypophyseal tracts causes atrophy and so loss of function of the neurohypophysis. The inference is compelling that, in animals polyuric after section of the supraoptico-hypophyseal tracts, there is absence of the antidiuretic hormone normally produced by the neuro-hypophysis. The polyuria is then explained as the release from the restraint normally imposed by the neurohypophysis in its humoral control of the secretion of urine.

Fisher, Ingram, and Ranson [1938] regard the neurohypophysis as consisting of three parts; the pars nervosa, the pituitary stalk, and the median eminence of the tuber cinereum, which all have a similar histo-logical structure. Accordingly, simple removal of the posterior lobe, leaving the stalk intact, does not result in permanent diabetes, because sufficient tissue of the neurohypophysis remains in the stalk and median eminence to restrain the rate of urine secretion; on the other hand this tissue also atrophies when the tracts are sectioned sufficiently far forward and so diabetes results. A correlation was therefore expected between the daily urine volume of dogs after removal of the posterior lobe or section of the supraoptico-hypophyseal tracts and the size of the residual inhibition produced by faradic stimulation. The data from this series and the previous paper [O'Connor and Verney, 1942] are collected in Table I and show no such correlation, the residual inhibition being of the order of 5 per cent. of its preoperative size in both groups of animals. However, Table I gives grounds for doubting whether the residual inhibition is a measure of the amount of functional tissue remaining in the neurohypophysis. "Tanner I" was the animal in which the section of the stalk was found at post-mortem to be incomplete and in which nerve fibres were found reaching the pars nervosa, yet the residual inhibition by emotional stress was only 2 per cent. of the preoperative inhibition. By this criterion the loss of neurohypophysis was more

TABLE I.

Operation.	Dog.	Daily urine volume 3–6 weeks after operation.	Inhibition of water-diuresis by emotional stress.
Section of supra-optic tracts	Span	320	less than 6
	Tanner I	150	2
	Hobo	500	7
	Tanner II	200	2
Removal of the posterior lobe of the pituitary	Spick	110	7
	Sin	170	less than 10
	Tarzan	100	3
	Scatterbrain	100	5

In columns 3 and 4 the figures are expressed as percentage of the preoperative value for that animal.

complete than that suffered by "Hobo", where the stalk was completely severed and a good polyuria followed. It appears then that the residual inhibition by emotional stress may not be an accurate measure of the small residual function of the neurohypophysis following operative procedures, and other methods will be required to establish by direct evidence that there is a significant residual function of the neurohypophysis to account for the absence of diabetes following removal of the posterior lobe.

SUMMARY.

1. Section of the supraoptico-hypophyseal tracts in four dogs resulted in atrophy of the neurohypophysis and a varying degree of diabetes insipidus.

2. After section of the supraoptico-hypophyseal tracts, a greatly reduced amount of antidiuretic substance was released from the neuro-hypophysis during emotional stress, as was shown by an almost complete abolition of the slow type of inhibition of water-diuresis. The amount of antidiuretic substance released was approximately 5 per cent. of the amount liberated in response to the same stimulus before tract section.

3. There was no difference in the size of the residual inhibition by emotional stress after tract section and after removal of the posterior lobe of the pituitary.

4. The results are discussed in relation to the production of diabetes insipidus by section of the supraoptico-hypophyseal tracts.

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A STUDY OF SOME CONDITIONS INFLUENCING THE RATE OF EXCHANGE OF OXYGEN IN BLOOD *IN VITRO*. By J. FEGLER and JEAN BANISTER. From the Physiology Department, Edinburgh University.

INTRODUCTION.

BARCROFT and HILL [1910] were the first to estimate the velocity of release of oxygen from a solution of hæmoglobin. They bubbled a stream of nitrogen through the solution and noted a speeding up of the reaction with an increase of temperature. Later Mathison [1911] used a modification of the bubbling method for measurement of the velocity of release of oxygen from defibrinated blood, and showed that the addition of carbon dioxide or other acids to the blood increased the velocity of the process. Oinuma [1911], working with Mathison's method, measured the relative rates of uptake and release of oxygen in defibrinated blood. He used hydrogen to reduce the blood and equilibrated it with oxygen at a partial pressure of 100 mm. Hg for measurements of the rate of uptake and reversed the procedure for the measurement of release. Oinuma's experiments indicated that the velocities of uptake and release of oxygen tended to be the same at 37·5° C., and partial pressures of oxygen of 100 mm. Hg and of carbon dioxide of 40 mm. Hg. Koeller [1923], working with a colorimetric method, measured the rates of oxygenation and reduction of the blood at 38° C. He confirmed Oinuma's finding that the rates were equal at this temperature and under the influence of carbon dioxide or other acids added to the blood. His results also confirmed Oinuma's observations on the effect on the velocity of oxygenation of blood of exposure to different partial pressures of oxygen.

Hartridge and Roughton [1923, 1925, 1926] published a series of papers containing their results on velocity of oxygenation and reduction of diluted solutions of laked blood. Their ingenious method enabled them to measure the absolute time factor involved in the reduction of oxyhæmoglobin and oxygenation of reduced hæmoglobin. They achieved almost instantaneous mixing of their oxyhæmoglobin solution with the reducing solution of sodium hydrosulphite in a specially constructed chamber. The mixture was then forced at constant rate

through a glass tube and the percentage of oxyhæmoglobin estimated at different sectors, with Hartridge's reversion spectroscope. The reduction of oxyhæmoglobin was found to be complete in about 0·025–0·125 seconds, increase of temperature and hydrogen ion concentration shortening the time of the reaction.

The reaction time for oxygenation of the hæmoglobin solution was found to be shorter than that for the reduction and was scarcely influenced by variations in temperature or hydrogen ion concentration. Hartridge and Roughton [1926] and Roughton [1932] then continued their experiments using diluted red blood cell suspensions. They studied the velocity of redistribution of oxygen between the oxygenating medium and the cells. They found that the uptake of oxygen by cell suspensions was about ten times slower than the uptake of oxygen by a hæmoglobin solution of equivalent concentration. They discussed the rate of diffusion through the cell membrane as well as through successive layers of cytoplasm within the red cells. They considered that diffusion through the layers of the medium in which the cells were suspended could not be responsible for the slowing of the reaction time as the partial pressure of the gas in the medium was found to be constant throughout; but that diffusion through the red-cell membrane and diffusion through the layers of cytoplasm within the red cell were probably the factors connected with the slowing of the reactions.

Millikan [1936], using Hartridge and Roughton's mixing chamber, but a photo-electric colorimeter instead of a reversion spectroscope, compared the velocity of oxygen in muscle hæmoglobin solutions and hæmoglobin solutions. In general his results confirmed those of Hartridge and Roughton on hæmoglobin solutions, but ·the muscle hæmoglobin reactions tended to be quicker on the whole. Dirken and Mook [1931], working on red-cell suspensions in serum, used a chamber similar to Hartridge and Roughton's to obtain instantaneous mixing of the cells and serum. Then, after appropriate time intervals, they separated the two by filtration, and analysed the serum. Their results in general confirmed those of Hartridge and Roughton. McEllroy and Guthrie [1927], using a manometric method, estimated the velocity of oxygenation of blood reduced to about 70 p.c. of its oxygen capacity *in vacuo* of man, dog, pig, and rabbit. They obtained complete saturation with oxygen in 4–6 minutes and found that variations in hydrogen ion concentration did not have a great effect on the time of the reaction. They observed that diluting the blood with serum or saline reduced the time of saturation and mentioned the possible importance of variations in viscosity.

The main purpose of our investigations at earlier periods was to find the effect of variations in the concentration of red cells in whole blood on the velocity of exchange of the blood gases, and also to assess the importance of the mutual relations between the rates of exchange of

oxygen and of carbon dioxide. Thus a method had to be devised which would permit the necessary measurements. Bubbling gas mixtures through the blood would have been difficult to adapt for use with concentrated whole blood, owing to excessive frothing. The mixing chambers used by Hartridge and Roughton and by others probably would have produced less satisfactory mixing of the more concentrated suspensions. The colorimetric method would not allow estimation of CO_2 exchange. Some experiments were attempted with the manometric method as used for measurements of velocity of CO_2 exchange in whole blood [Fegler, 1944], but this excluded simultaneous measurements of carbon dioxide and oxygen exchanges. Mathison had considered the possibilities of a tonometric method but discarded it after some trials owing to technical difficulties. However, after many attempts it was found possible to design a special tonometer which overcame most of the disadvantages of the earlier methods.

METHODS.

Blood Analysis.—The relative volume of red cells was estimated by the method described by Meyerstein [1942]. For each determination in duplicate the blood was spun in capillary tubes at 10,000 revolutions per minute for fifteen minutes exactly. The combined sampling and reading error was less than ± 0.5 p.c. of the absolute value.

Hæmoglobin determinations in duplicate were made using a photo-electric comparison of the concentration of hæmoglobin after conversion to cyanhæmoglobin as described by Stadie [1920] and modified by Wu [1922]. The photometer used was the Hilger-Spekker model.

For estimations of the blood and plasma viscosity a low velocity type viscometer of Oswald pattern was used, with a capillary bore of 0.8–1.0 mm. 4.0 c.c. only of fluid was required for the measurement which was made in a water-bath, the temperature of which was controlled to within $\pm 0.2°$ C. Readings on any one sample could be reproduced to a coefficient of variation of less than 1.0. Samples of the original blood or blood after equilibration with the initial gas mixture were used for the determinations. The data given in Table II are expressed as the relative viscosity, which is the ratio of the time for blood or plasma to the time for distilled water at the same temperature.

The determinations of pH of blood were made after equilibration at given temperatures with nitrogen and 6 p.c. carbon dioxide in a tonometer; the pH values were taken from the nomogram given by Peters and Van Slyke [1932] after analysis of the gas mixture in the tonometer and of the carbon dioxide content of the blood.

The rate of carbon dioxide uptake and oxygen release was measured by exposing blood previously saturated with pure oxygen to a gas mixture of nitrogen and 6 p.c. carbon dioxide (hereafter called N/C

through a glass tube and the percentage of oxyhæmoglobin estimated at different sectors, with Hartridge's reversion spectroscope. The reduction of oxyhæmoglobin was found to be complete in about 0·025–0·125 seconds, increase of temperature and hydrogen ion concentration shortening the time of the reaction.

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whole manipulation (including the tilting of the tonometer) was completed in 20 seconds.

The precision of this method is shown by the figures in Table I and seems to be satisfactory.

The treatment of the blood described above caused slight hæmolysis and some drying as determined by hæmatocrit, hæmoglobin, and
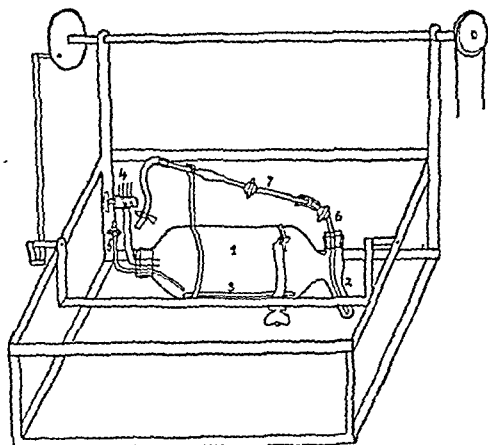
Fig. 1 is an illustration of the "glass rod tonometer" placed on its rocking frame. 1, the tonometer of about 290 c.c. capacity. 2, sealed-on glass tube for collection of blood samples. 3, glass rod. 4, two-way tap for filling with gas mixtures. 5, needle for introduction of blood samples. 6, needle for withdrawal of blood samples. 7, Ostwald pipette.

viscosity measurements. The relative degree of hæmolysis between samples was judged by inspection of the plasma layer in the hæmatocrit tube and was not found to be severe enough to influence significantly the ratio mgm. Haem Ferrous/vol. of R.B.C. In some experiments no increase in hæmolysis compared with the control sample was observed. The variations which occurred appeared not to affect the velocity of oxygen exchange. The influence of small amounts of carbonic anhydrase in the plasma, resulting from hæmolysis, upon the kinetics of carbon dioxide exchange in oxalated whole blood is in course of investigation and the result will be described in a later communication.

RESULTS.

The Character of the Velocity Curves.

When blood equilibrated with pure oxygen or the N/C mixture was exposed to the N/C mixture or pure oxygen respectively for different periods of time, the velocities of the carbon dioxide and oxygen exchanges were found to diminish with time as represented in fig. 2.

When the logarithm of the differences between 100 p.c. saturation

mixture) for one minute in the tonometer in a proportion of 6·0 c.c. of blood to about 290 c.c. of gas mixture. Similarly the rate of oxygen uptake and carbon dioxide release was measured by exposing reduced blood, equilibrated with the N/C mixture, to pure oxygen in the same proportions for the same length of time. Various other times of exposure were also tested in some experiments enabling a curve to be constructed showing the course of the process.

Samples of the blood before and after exposure were analysed for their oxygen and carbon dioxide content by the method for the simultaneous estimation of these gases in 1·00 c.c. of blood in a Van Slyke-Neil constant volume manometric apparatus described by Peters and Van Slyke [1932].

Preparation of Blood Samples.—Blood was collected from a slaughtered ox or sheep directly into a flask containing enough solid potassium oxalate to give a concentration in the blood of about 0·3 p.c. It was filtered through glass wool and measured. At this stage variations in the ratio mgms. of ferrous Haem per 100 c.c. of blood/red-cell volume p.c. were determined in twenty-five samples. The value obtained was 1·33 with a standard deviation of 0·06. When the relative red-cell volume required alteration plasma and cells were separated by centrifuging and one or the other added to the whole blood to give the required volume of red cells. Usually 60 c.c. portions of the blood were exposed to a vacuum in Buchner flasks of 2-litre capacity two or three times to rid them of oxygen and carbon dioxide; they were then equilibrated for one hour at room temperature with the required gas mixtures. The oxygen and N/C mixtures were commercially prepared in gas cylinders and their composition checked by analysis. After equilibration the blood was stored over mercury at 4° C. until used. Samples were brought to the required experimental temperature in a water-bath about ten minutes before introduction into the tonometer.

Gaseous Equilibration of Blood.—The "glass rod" tonometer which was used is illustrated in fig. 1. It was evacuated and filled several times with the required gas mixture *via* a two-way tap before use. After placing the tonometer on its frame in the water-bath the pressures were equalised; then the 6·0 c.c. blood sample was introduced by syringe into the tonometer which was immediately set rocking in its long axis at a constant speed and excursion. The glass rod moving within the tonometer distributed a thin layer of blood evenly over a constant surface.

At the end of the required period of exposure the reaction between gas mixture and blood was stopped within 5 seconds, by tilting the apparatus vertically through 90° C. In this position the blood flowed quickly into the small tube 2, fig. 1, so that only a small surface was in contact with the gas mixture. The 1 c.c. sample for analysis was withdrawn into the Ostwald pipette from the bottom of the tube. The

or desaturation of the blood with oxygen or carbon dioxide and the percentage saturation found after exposure (the formulæ used for calculation of the percentages is given in the Appendix) was plotted against the time of exposure a straight line was obtained. (fig. 2, dotted
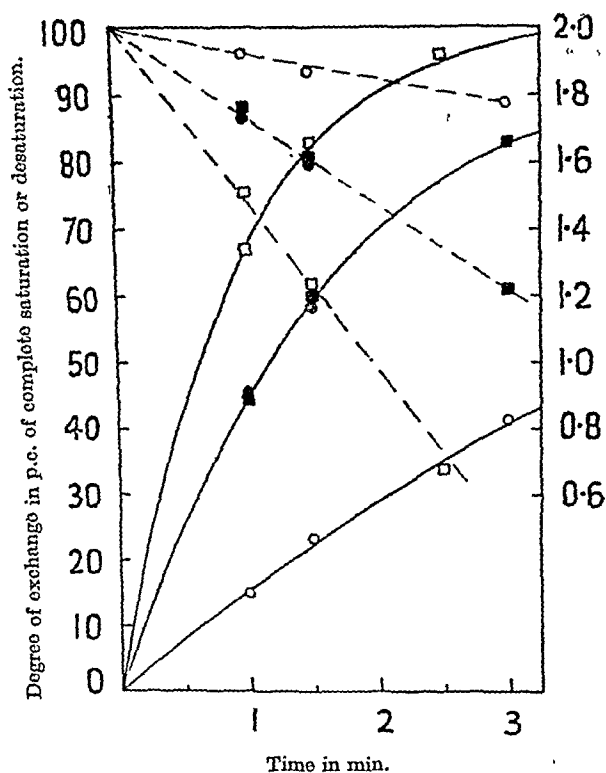
Fig. 2 shows the velocity curves for the uptake and release of oxygen and carbon dioxide in oxalated ox or sheep blood exposed to pure oxygen or nitrogen and 6 p.c. carbon dioxide at 38° C.   The interrupted lines are logarithmic.

□ =oxygen uptake.   O =oxygen release.
▥ =carbon dioxide uptake.   ● =carbon dioxide release.

lines).   This showed the original curves to be exponential in character and they corresponded to the equation

$$a - x = a \cdot 10^{-kt}, \tag{1}$$

where $a = 100$ p.c. saturation or desaturation of the blood.

$x =$ percentage saturation found after exposure.

$k =$ velocity constant for the process.

$t =$ time of exposure in minutes.

From equation (1) $k = \dfrac{\log a - \log (a - x)}{t}.$ (2)

Date of Exp.	Blood saturated in O_2, exposed to N_2 + 6 p.c. CO_2.						Blood reduced in N_2 + 6 p.c. CO_2, exposed to O_2.					
	Initial compos.		Compos. after 1 min. exposure.		Velocity of CO_2 uptake and O_2 release in p.c. of total cap.		Initial compos.		Composition after 1 min. exposure.		Velocity of CO_2 release and O_2 uptake in p.c. of total cap.	
	CO_2 V. p.c.	O_2 V. p.c.	CO_2 V. p.c.	O_2 V. p.c.	CO_2	O_2	CO_2 V. p.c.	O_2 V. p.c.	CO_2 V. p.c.	O_2 V. p.c.	CO_2	O_2
29.8.44 Ox blood 25° C.	15·92	22·70	38·33	19·37	35·10	17·40	79·85	3·59	53·07	14·80	41·91	58·00
	..	..	38·40	19·45	35·16	17·00	..	..	53·15	14·60	41·76	57·60
	..	..	38·36	19·33	35·10	17·63	..	..	52·80	14·70	42·30	58·10
Mean			38·36	19·38	35·12	17·34			53·01	14·70	41·99	58·10
Max. Deviation			±0·04	−0·05 +0·07	−0·02 0·04	−0·34 +0·29			−0·21 +0·14	0·10	−0·23 +0·31	±0·50
7.8.44 Ox blood 38° C.	8·79	22·38	27·68	17·76	44·37	21·30	51·36	0·70	35·54	14·23	37·16	62·40
	..	..	27·35	18·16	43·00	19·50	..	..	34·02	15·09	40·70	66·30
	..	..	26·02	17·70	41·83	21·30	..	..	34·74	14·09	39·00	61·76
Mean			27·22	17·80	43·27	20·70			34·76	14·47	38·95	63·49
Max. Deviation			−0·6 +0·46	−0·13 +0·27	−1·44 +1·10	−1·20 +0·60			−0·74 +0·78	−0·38 +0·62	−1·79 +1·75	−1·73 +2·81
21.8.44 Ox blood 41° C.	24·36	22·05	38·07	16·91	34·78	24·83	63·77	1·35	36·67	18·86	68·70	84·59
	..	..	37·70	16·63	33·84	26·18	..	..	35·58	18·22	71·53	81·50
	..	..	38·70	16·84	36·37	25·17	..	..			..	..
Mean			38·37	16·79	35·00	25·39			36·13	18·51	70·15	83·05
Max. Deviation			−0·67 +0·33	−0·16 +0·12	−1·16 +1·37	−0·56 +0·79			±0·55	±0·32	±1·39	±1·55

TABLE II.

Exp. No.	Temperature of the bath, °C.	Relative red-cell volume.	Haemoglobin in mgms. p.c. of Haem. Fe.	pH.	Viscosity.	Oxygen content in reduced blood, V. p.c.	Oxygen content in reduced blood after exposure for 1 min. to O₂, V. p.c.	Oxygen content in oxygenated blood in V. p.c.	Oxygen content in oxygenated blood after exposure for 1 min. to N₂ + 6 p.c. CO₂, V. p.c.	Oxygen uptake.	Oxygen release.	Remarks.
17	17	40·3	57·5	7·44	6·80	1·12	15·36	23·40	20·60	50·0	10·9	
21 (a)	18·5	40·2	53·0	··	6·08	3·10	14·30	22·83	··	56·7	··	
32	20	42·5	··	··	··	1·84	11·15	22·66	19·90	44·7	13·3	
34a	20	40·8	54·0	7·50	5·76	0·86	12·99	22·09	19·92	57·1	10·2	
34	25	41·9	··	7·38	6·38	2·83	12·08	20·94	18·72	54·0	12·3	
22	30	40·1	54·0	··	4·90	0·62	14·29	20·46	18·63	62·5	17·5	
23	30	40	··	7·38	··	1·47	··	23·48	19·91	··	16·2	
34b	30	40·8	54·0	7·40	4·64	0·74	14·38	21·96	18·53	64·2	16·2	
6	36	··	60·0	··	··	0·80	··	21·81	18·00	··	18·1	
2a	38	32·1	46·0	··	2·78	0·49	··	18·59	13·89	··	25·9	Plasma diluted with Tyrode.
,, b	,,	33·5	··	··	2·41	0·74	··	18·33	13·40	··	28·5	,,
,, c	,,	32·5	··	··	3·37	0·49	··	17·20	13·30	··	23·3	
4	,,	33·0	··	··	··	0·25	··	19·23	14·80	··	23·3	
9	,,	40·5	56·3	7·38	4·03	0·61	··	22·27	18·80	65·8	16·0	
21 (b)	,,	40·2	53·0	7·38	3·10	0·62	14·90	22·30	10·66	82·0	26·3	
5	,,	23·7	··	7·43	6·54	0·74	11·78	14·20	25·67	48·6	0·2	
8a	,,	55·0	··	7·43	4·37	1·96	15·20	29·17	18·60	68·0	18·4	
,, b	,,	40·0	··	··	2·84	2·20	15·93	22·30	11·07	81·3	24·4	
,, c	,,	24·8	··	7·34	··	0·85	11·84	14·37	18·16	61·4	19·2	
,, 7	,,	40·2	··	7·43	··	0·74	14·09	22·33	17·20	64·6	23·0	
8	,,	38·9	··	7·41	··	0·25	14·51	22·30	17·20	67·0	22·2	
9	,,	38·5	··	··	··	0·49	14·90	21·97	19·06	66·7	15·7	
3	,,	41·1	··	··	4·39	1·70	15·43	22·30	17·04	··	23·6	
,, 6	40	··	55·0	··	··	1·78	··	22·53	··	75·3	··	
4	,,	··	57·0	··	··	1·29	16·56	21·57	16·54	··	27·3	
,,	41	··	60·5	7·34	··	0·08	··	22·40	18·05	··	23·7	
,,	,,	··	56·0	··	··	2·30	16·57	22·96	··	75·0	··	
,, 0	,,	38·6	54·5	··	3·90	0·49	13·67	19·53	15·01	69·2	23·7	

From equation (2), using one experimental point (1 minute 30 seconds), the velocity constants for the release and uptake of oxygen and carbon dioxide were calculated and values as follows obtained:—

carbon dioxide uptake, $k = 0.2640$; carbon dioxide release, $k = 0.2610$; oxygen uptake, $k = 0.4923$; oxygen release, $k = 0.0747$.

The theoretical curves calculated from these values of k for different time intervals corresponded closely to the points found by experiment.

The curves shown in fig. 2 and the difference in the values of the velocity constants for oxygen exchange are some indication of the marked inequality between the rate of oxygen uptake by blood equilibrated with the N/C mixture and exposed to pure oxygen, and the rate of oxygen release by blood equilibrated with oxygen at one atmosphere and exposed to the N/C mixture. Fig. 2 shows that for carbon dioxide the rates of uptake and release tended to be similar to each other, although some results obtained showed that the rate of uptake of carbon dioxide might be quicker than its rate of release.

The remainder of this paper deals with the experimental results obtained for oxygen exchange only. The data relating to the problem of carbon dioxide exchange and to the interrelation between the rates of oxygen and carbon dioxide exchange will be described in a later communication.

The Influence of Variations in Temperature, Relative Red-Cell Volume, and Relative Viscosity of the Blood on the Rate of Oxygen Exchange.

Temperature.—The blood samples used for these experiments were of similar red-cell volumes (38–42 p.c.). The normal procedure was followed, the gas mixture chosen being pure oxygen and the N/C mixture. The time of exposure of the blood to the gas phase was one minute. The temperature of the water-bath was controlled to within $\pm 0.5°$ C. and the range covered was from 17–41° C.

The results of the experiments are given in Table II (experiments Nos. 17, 21 *a* and *b*, 24 *a* and *b*, 34, 22, 23, 19, 26, 27, 28, 29, 35, 3, 6, 4, 30) and are illustrated by the scatter diagram fig. 3.

The relation found between the rate of oxygen exchange and the experimental temperature chosen is illustrated by the regression lines in fig. 3 (contin.). The regression lines were used as the basis for the calculation of the velocity constants over the range of temperature tested and the values of these, plotted against temperature, are also shown in fig. 3 (dotted lines). The curves for uptake and release are similar in character and indicate that the rate of both processes was augmented when the temperature was increased; the difference in slope between the curves suggesting a greater effect of temperature upon the rate of release of oxygen than upon its uptake. The latter observation confirms in principle the results obtained by Hartridge and Roughton

Relative Red-Cell Volume.—Blood samples of different red-cell volumes were prepared in the usual way and exposed to the gas phase for one minute at 38° C. The gas mixtures used for saturation and exposure were pure oxygen and the N/C mixture.

The results of the experiments are given in Table II (experiments
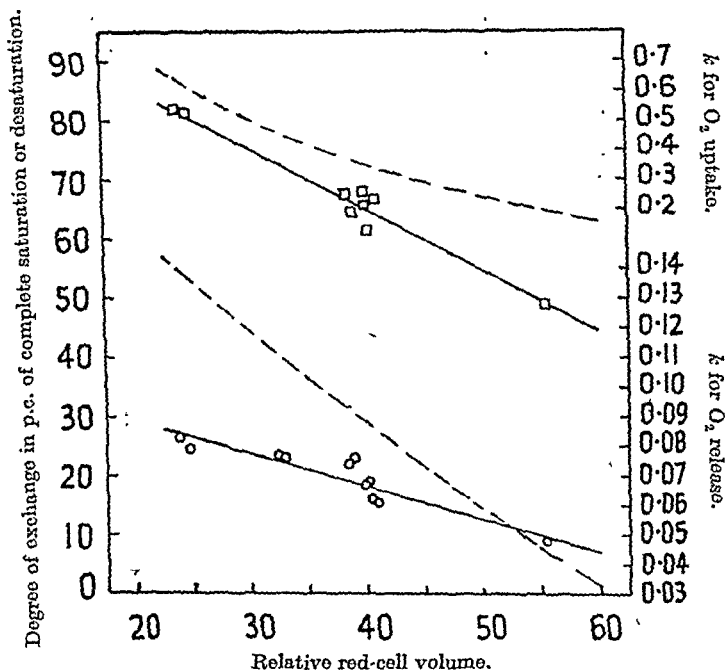
Fig. 4 shows the effect of variations in relative red-cell volume upon the rates of uptake and release of oxygen in blood exposed to pure oxygen or nitrogen and 6 p.c. carbon dioxide for one minute at 38° C. The continuous lines are the regression lines and the interrupted lines are the velocity constants.

□ = oxygen uptake.   O = oxygen release.

Nos. 12 *c*, 14, 19, 21, 25, 26 *a*, *b*, *c*, 27, 28, 29, 35) and are illustrated by the scatter diagram fig. 4.

The regression lines show the linear relation between the velocities of oxygen uptake and release and the relative red-cell volume, the velocities of exchange decreasing as the relative red-cell volume was increased. The regression lines were used for the calculation of the velocity constants and the values of these for different relative red-cell volumes are shown by fig. 4 (dotted lines). The difference in slope between the two velocity constant lines indicates that the relative red-cell volume of the blood has a greater effect upon the velocity of oxygen release than upon uptake.

Relative Viscosity of the Blood.—The factors contributing to the viscosity of whole blood have been investigated by several workers.

[1923, 1925, 1926], who found that although the effect of temperature upon the velocity of release of oxygen was very marked, its effect upon the velocity of oxygen uptake was negligible. A possible explanation for the lack of agreement between our results and theirs in respect of the uptake of oxygen may lie in the fact that Hartridge and Roughton
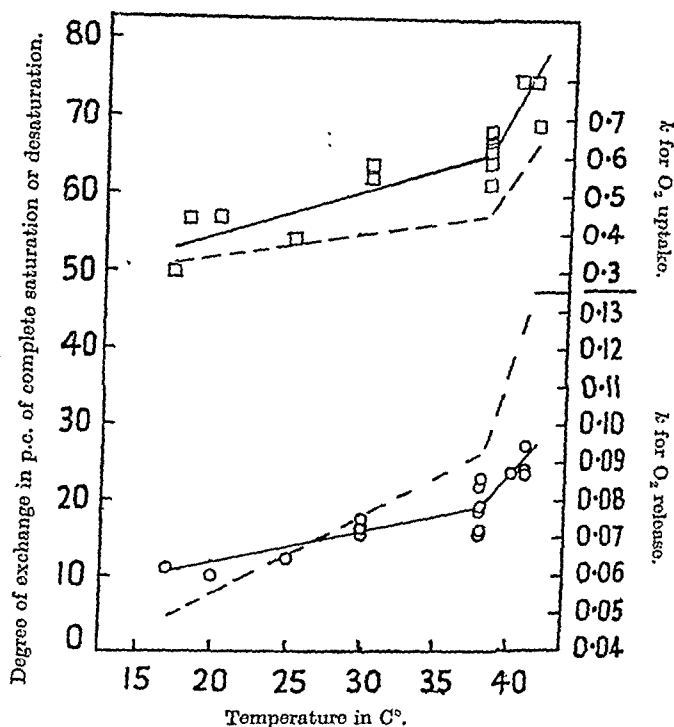
Fig. 3. shows the effect of variation in temperature upon the rates of uptake and release of oxygen in blood of a relative red-cell volume of about 40 p.c. exposed to pure oxygen or nitrogen and 6 p.c. carbon dioxide for one minute. The continuous lines are the regression lines and the interrupted lines are the velocity constants.

□ =oxygen uptake.   ○ =oxygen release.

worked with dilute hæmoglobin solutions or red-cell suspensions, whereas we used whole blood.

In general the temperature effect upon the rate of both processes was less marked at the lower temperatures than at the higher temperatures tested; this observation is illustrated by reference to the change in temperature coefficients for the reaction. From 17–37° C. the temperature coefficient for the uptake of oxygen was 1·30–1·40 and for the release of oxygen was 1·15–1·20, values which are characteristic of physical processes. Above 38° C. values of 2·31–2·34 for both uptake and release were obtained and these approximate more nearly to coefficients found for chemical reactions.

uptake and release emphasises that changes in the relative viscosity have a greater effect upon the rate of release of oxygen than upon its uptake.

Fig. 6 shows the velocity constants for oxygen exchange plotted against relative viscosity, relative red-cell volume, and temperature

FIG. 6 shows the alterations in value of the velocity constants for the uptake and release of oxygen according to the relative viscosity of the blood and the associated temperature and relative red-cell volume changes. The abscissa has been subdivided in order to represent the changes in the relative red-cell volume at 38° C., and the variations in the temperature of the blood or a relative red-cell volume of about 40 p.c., which were found experimentally to cause alterations in the relative viscosity of the blood equivalent to the values indicated on the basic scale. The velocity constant values were taken from graphs 3, 4, 5 and were plotted separately for each variable. The upper row of points represents the release of oxygen and the lower row of points the uptake of oxygen.

O = temperature.   ● = relative viscosity.   ⊗ = relative red-cell volume.

of blood. The abscissa scales for temperature and red-cell volume have been arbitrarily adjusted to show the correlation found by experiment between these two factors and viscosity which is plotted on a linear abscissa scale. The values of the velocity constants for the three variables correspond closely to each other for any one temperature within the range 20–38° C. Above a temperature of 38° C. the temperarure effect upon the value of the velocity constants is shown to be significantly greater than that produced by a proportionate reduction in the relative viscosity of the blood. It appears, however, that an explanation for the effects of temperature between 20–38° C., and the effects of changes in the relative red-cell volume on the rates of exchange

Expressions for the relative effect of red-cell volume and plasma viscosity on whole blood viscosity have been worked out by Trevan [1918] and Whittaker and Winton [1933]. The effect of temperature variations on the relative viscosity of whole blood was shown to be considerable by Rothlin [1920]. His observations have been confirmed by us within the range tested 18–41° C., but under different experimental conditions.

Viscosity measurements were made on samples of the blood used for
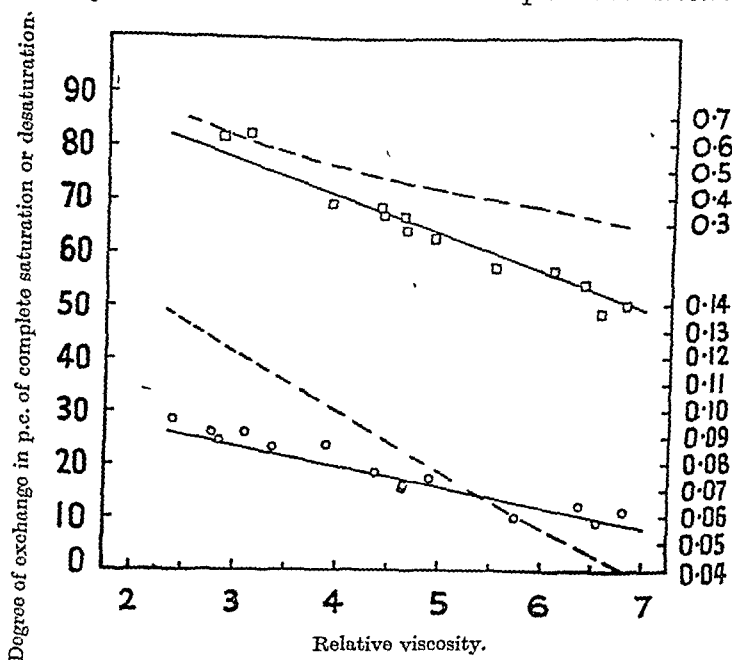
FIG. 5 shows the effect of variations in the relative viscosity upon the rates of uptake and release of oxygen in blood exposed to pure oxygen or nitrogen and 6 p.c. carbon dioxide for one minute. The continuous lines are the regression lines and the interrupted lines are velocity constants.

□ =oxygen uptake.   O =oxygen release.

the oxygen exchange determinations in order to investigate whether the effects of alterations in temperature and relative red-cell volume on the rates of exchange previously mentioned could be ascribed to the concomitant changes in the relative blood viscosity.

The results are given in Table II (experiments Nos. 17, 21, 24 a, 34, 22, 24 b, 12 a, b, c, 21, 25, 26 a, b, c, 35, 30) and are illustrated by the scatter diagram of fig. 5.

The linear relation between the relative viscosity of the blood and its rate of oxygen exchange is clearly shown by the regression lines in fig. 5 (contin.). The velocity constants were calculated from the regression lines, and their values are plotted against the relative viscosity in fig. 5 (dotted lines). The difference in slope between these lines for

uptake and release emphasises that changes in the relative viscosity have a greater effect upon the rate of release of oxygen than upon its uptake.

Fig. 6 shows the velocity constants for oxygen exchange plotted against relative viscosity, relative red-cell volume, and temperature
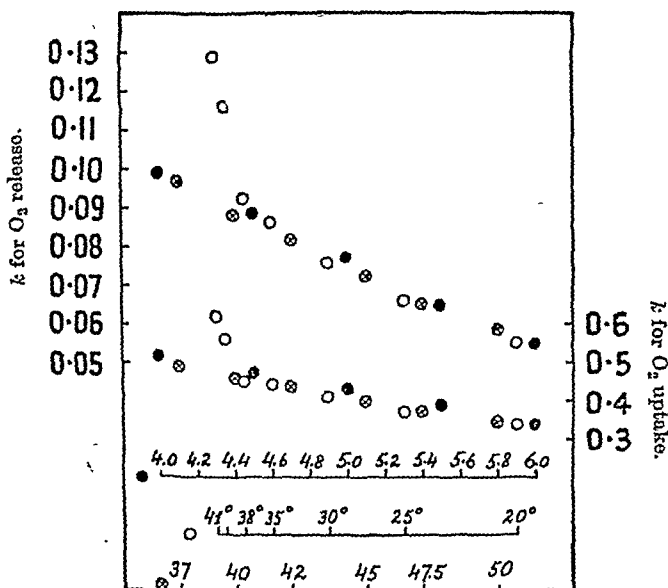

Fig. 6 shows the alterations in value of the velocity constants for the uptake and release of oxygen according to the relative viscosity of the blood and the associated temperature and relative red-cell volume changes. The abscissa has been subdivided in order to represent the changes in the relative red-cell volume at 38° C., and the variations in the temperature of the blood or a relative red-cell volume of about 40 p.c., which were found experimentally to cause alterations in the relative viscosity of the blood equivalent to the values indicated on the basic scale. The velocity constant values were taken from graphs 3, 4, 5 and were plotted separately for each variable. The upper row of points represents the release of oxygen and the lower row of points the uptake of oxygen.

O = temperature.   ● = relative viscosity.   ⊗ = relative red-cell volume.

of blood. The abscissa scales for temperature and red-cell volume have been arbitrarily adjusted to show the correlation found by experiment between these two factors and viscosity which is plotted on a linear abscissa scale. The values of the velocity constants for the three variables correspond closely to each other for any one temperature within the range 20–38° C. Above a temperature of 38° C. the temperature effect upon the value of the velocity constants is shown to be significantly greater than that produced by a proportionate reduction in the relative viscosity of the blood. It appears, however, that an explanation for the effects of temperature between 20–38° C., and the effects of changes in the relative red-cell volume on the rates of exchange

TABLE III.

p.p. O$_2$ mm. Hg.	Velocity of O$_2$ uptake.						Velocity of O$_2$ release.					
	Exp. 1.		Exp. 2.		Exp. 3.		Exp. 1.		Exp. 2.		Exp. 3.	
	p.c. of total cap.	K.	p.c. of total cap.	K.	p.c. of total cap.	K.	p.c. of total cap.	K.	p.c. of total cap.	K.	p.c. of total cap.	K.
31–33	4·5	0·020	..	..	..	..	11·0	0·051	15·4	0·073	6·9	0·031
105–108	15·4	0·073	19·2	0·093	18·6	0·089	13·9	0·065	18·8	0·090	8·9	0·041
150	..	..	26·5	0·134	..	..	..	..	..	..	..	..
200	..	..	..	..	..	..	..	..	21·4	0·105	..	..
250–258	..	..	37·3	0·203	..	..	..	..	..	..	11·3	0·052
436	43·5	0·248	..	..	..	..	13·4	0·063	..	..	..	..
510	..	..	..	..	51·2	0·312	..	..	..	..	..	..
740–760	59·0	0·387	63·3	0·435	72·0	0·553	..	..	21·9	0·107	11·4	0·053

of oxygen by the blood, may be found in the resulting alterations in the relative blood viscosity.

The Effect of Exposure to or Saturation with Partial Pressures of Oxygen less than one Atmosphere upon the Velocities of Exchange of Oxygen by the Blood.

The effect of reducing the pressure head of oxygen on the rates of oxygen exchange by the blood was found by experiments in which the
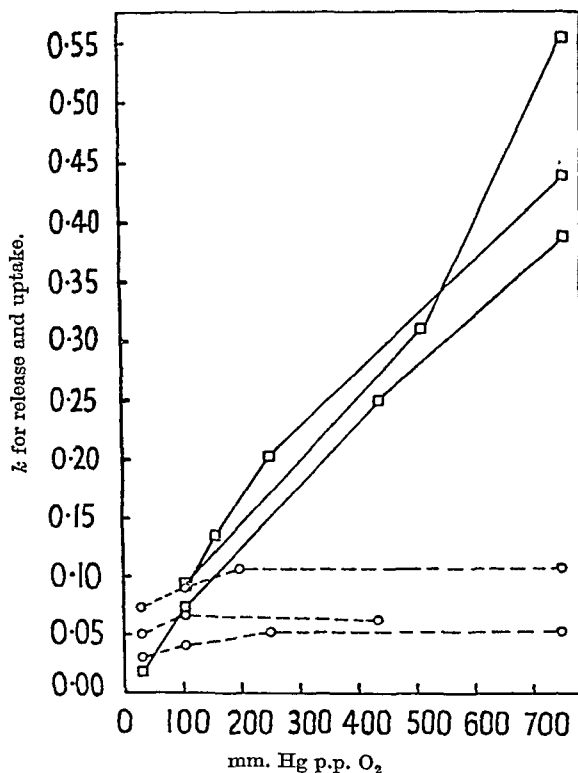
FIG. 7 shows the variations of the velocity constants at 38° C. (*a*) for the uptake of oxygen in blood equilibrated with nitrogen and 6 p.c. carbon dioxide and then exposed to different partial pressures of oxygen, and (*b*) for the release of oxygen from blood equilibrated with different partial pressures of oxygen and then exposed to nitrogen and 6 p.c. carbon dioxide at one atmosphere pressure.

□ =oxygen uptake.   O =oxygen release.

normal procedure was followed, but instead of pure oxygen, mixtures of nitrogen and oxygen giving partial pressures of 760–30 mm. Hg of oxygen were used. The time of exposure was one minute and the temperature 38° C.

The results are given in Table III.

Fig. 7 illustrates the results given in Table III.

The above illustration indicates that there is a marked qualitative and quantitative difference between the influence of the partial pressure of oxygen within the blood on the velocity of its release, and of the partial pressure of the gas phase outside the blood on the velocity of its uptake. There is an almost linear relationship between the rate of oxygen uptake by the blood and the partial pressure of oxygen to which it is exposed. Although increasing the partial pressures of oxygen within the blood
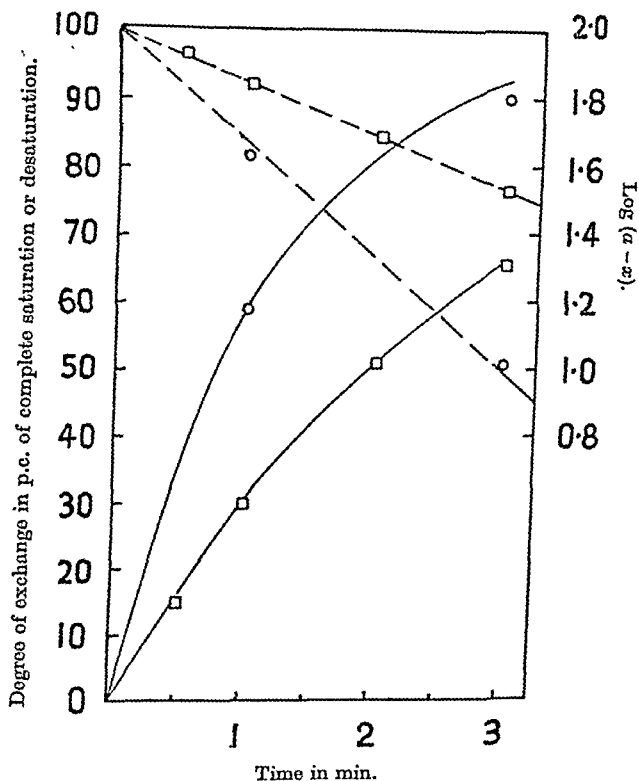
FIG. 8 shows the rate of oxygen uptake and release in blood with intracellularly inactivated hæmoglobin at 38° C. and exposed to pure oxygen or nitrogen and 6 p.c. carbon dioxide.

□ = oxygen uptake.　　　O = oxygen release.

from 30–250 mm. Hg tended to increase the rate of its release, above this level the rate remained comparatively constant.

The results also show that, at 38° C. and at partial pressures of oxygen and carbon dioxide similar to those found in the alveolar air, the rates of oxygen uptake and release by the blood tend to become equal. As the partial pressure of oxygen is raised above 100 mm. Hg the increase in the rate of oxygen uptake becomes progressively greater than that of release at least up to one atmosphere.

In connection with these results, experiments were made in which the

velocity of oxygen exchange was measured in blood with intracellularly inactivated hæmoglobin. Methæmoglobin was produced within the red cells by suspending them for one hour in isotonic (1·06 p.c.) solutions of sodium nitrite. After washing in 0·9 p.c. sodium chloride solution the methæmoglobin cells were resuspended in plasma to give a red-cell volume of 40 p.c. This suspension was then saturated with the oxygen or the N/C mixture and exposed for different intervals of time to the N/C mixture or oxygen respectively.

The result of one of these experiments is shown in fig. 8.

The velocity of oxygen release in these circumstances was found to be more rapid than the velocity of oxygen uptake. Experiments performed on plasma under similar conditions gave comparable results showing the release of 90 p.c. and uptake of 84 p.c. of total capacity after a one-minute exposure to the appropriate gas mixture.

These observations indicate that active hæmoglobin played a decisive part in determining the difference between the rates of oxygen uptake and release. Its presence increases the velocity of oxygen uptake but retards the velocity of oxygen release within the appropriate pressure ranges.

DISCUSSION.

We measured oxygen uptake and release over periods of one minute or longer, during which time the uptake or release was still progressive, and examined in detail the influence of various physical factors upon the velocity of oxygen exchange in oxalated whole blood. Under the conditions of test there was only a relatively small surface of blood in contact with the gas medium and therefore it seemed probable that the velocity of access of oxygen to the hæmoglobin within the red cells would tend to be the main factor limiting the results. If the rate of penetration into the blood by oxygen were a governing factor then the velocity of uptake and release of the gas should vary with changes in the relative viscosity of the blood and the pressure head of oxygen available.

The relative viscosity of the blood was altered by dilution of plasma, by varying the relative red-cell volume of the blood and by varying the experimental temperature. A change in the relative red-cell volume was found to affect the speed of equilibration of oxygen to just that degree expected if the relative viscosity were the limiting factor. Similarly below 38° C. the influence of temperature on the velocity constants was that which would be anticipated if the primary effect of the temperature were upon the relative viscosity, above 38° C., however, this was not true.

It was found that the rate of oxygen uptake by the blood was almost directly proportional to the available pressure head of oxygen (Table III,

fig. 7). On the other hand the rate of oxygen release was dependent in a less simple way upon the pressure, and it was suggested by the experiments with methæmoglobin cells (fig. 8) that the affinity of hæmoglobin for oxygen might be responsible for this effect. In assessing the importance of this difference in response from a practical point of view, it should perhaps be mentioned that the results of experiments on the influence of carbon dioxide upon the velocity of exchange of oxygen in the blood, to be published later, show that carbon dioxide has a negligible effect upon the rate of oxygen uptake of blood exposed to one atmosphere of oxygen. Its action in greatly increasing the rate of oxygen release from the blood was confirmed.

In conclusion it appears that although the affinity of hæmoglobin for oxygen and the chemical conditions in the blood affecting this probably determine the scale of the results obtained, yet these results may be significantly influenced by physical factors connected with the conditions of experiment. The rate of oxygen uptake by the blood is shown to be greatly affected by the pressure gradient of oxygen to which the blood is exposed, but with the partial pressure of oxygen fixed at one atmosphere the most important variable remaining seems to be the viscosity of the blood. The rate of release of oxygen from the blood is influenced considerably by the relative viscosity, but changes in the partial pressure of oxygen within the blood above 250 mm. Hg have little effect.

SUMMARY.

A tonometer is illustrated which ensures constant conditions of exposure of blood to gas mixtures for any desired interval of time, making possible a simultaneous measurement of the velocity of exchange of oxygen and carbon dioxide in whole blood.

The influence of variations in temperature, red-cell volume p.c., the relative viscosity of the blood, and in the pressure gradient of oxygen between the blood and the gas phase on the rate of exchange of oxygen by oxalated blood *in vitro* was investigated. It was found that

1. An increase of temperature, or a reduction in the relative viscosity of the blood or in its relative red-cell volume augmented the rates of uptake and release of oxygen.

2. The increases in the rate of exchange observed with rises of temperature from 20–38° C., or with reductions in the relative red-cell volume corresponded to just such increases in the rate of exchange as would be expected from the resulting alterations in the relative viscosity of the blood.

3. The rate of oxygen uptake varied in almost direct proportion with the partial pressure of oxygen to which the blood was exposed. The rate of oxygen release did *not* so accurately reflect the pressure gradient of oxygen between the blood and the gas phase.

APPENDIX.

The rate of exchange of gas has been expressed by calculating the amount of gas taken up or released in unit time as a percentage of the total amount of the gas contained in the blood at full saturation or desaturation corrected according to the following formulæ:—

ACO_2 and AO_2 = composition in vol. p.c. of reduced blood equilibrated with nitrogen and 6 p.c. carbon dioxide.

aCO_2 and aO_2 = composition of reduced blood after exposure to pure oxygen.

BCO_2 and BO_2 = composition of blood equilibrated in pure oxygen.

bCO_2 and bO_2 = composition of blood equilibrated in pure oxygen after exposure to nitrogen and 6 p.c. carbon dioxide.

$$\text{Rate of oxygen release} = \frac{(BO_2 - AO_2) - (bO_2 - AO_2) \cdot 100}{BO_2 - AO_2}.$$

$$\text{Rate of oxygen uptake} = \frac{(aO_2 - AO_2) \cdot 100}{BO_2 - AO_2}.$$

$$\text{Rate of carbon dioxide release} = \frac{(ACO_2 - BCO_2) - (aCO_2 - BCO_2) \cdot 100}{ACO_2 - BCO_2}.$$

$$\text{Rate of carbon dioxide uptake} = \frac{(bCO_2 - BCO_2) \cdot 100}{ACO_2 - BCO_2}.$$

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THE ACTION OF HÆMOLYSINS ON NORMAL AND PATHOLOGICAL HUMAN ERYTHROCYTES. By MONTAGUE MAIZELS. From the South-Eastern Emergency Blood Supply Depot, Maidstone.

THE excessive hæmolysis found in certain anæmias must be due either to normal hæmolytic agents acting on specially susceptible erythrocytes of a defective type, or else to cells of normal resistance being exposed to the action of lytic agents present in unusual quantity or activity. Such agents might be complex cellular systems or relatively simple hæmolytic substances. In the latter case, where unidentified lysins act *in vivo* only a proportion of the cells is hæmolysed while the rest appears to be unaffected. It may be presumed, however, that the unhæmolysed cells have also combined with some of the circulating lysin and in this case their response to the addition of known lysins, either *in vivo* or *in vitro*, might be different from that shown by normal cells. So, too, hæmolysis in stored blood may be due to degeneration of the erythrocyte with loss of cohesion in the surface constituents or to disintegration brought about by the action of a lysin, itself developed during storage. Here also the erythrocyte might show qualitative or quantitative differences on exposure to known lysins. The present paper deals with the altered response of such cells to known lysins added *in vitro*.

It is probable that the combined effects of an unidentified lysin adsorbed *in vivo* and a known lysin added *in vitro* will be neither simple nor additive. For Ponder [1934 *a*] has shown that when lysins are mixed, their combined effects may be greater than, equal to, or less than the sum of their separate actions. So, too, Wilbur and Collier [1943] have found that saponin and lysolecithin when acting together are usually mutually inhibitory, while the present writer has found that, under his conditions of experiment, lysolipin, bile salts, and soap inhibit lysis by saponin but are not themselves inhibited by it, nor is any inhibition found between members of the lysolipin, bile salts, soap group. It therefore seemed probable that where erythrocytes either *in vivo* or *in vitro* had been exposed to members of the latter group and had adsorbed an amount of lysin insufficient to cause complete lysis, the affected cells would be more susceptible to this group and less susceptible to saponin, or in other words, that increased susceptibility to lysolipin,

bile salts, and soaps, with decreased susceptibility to saponin, might be used as a test for previous exposure to lysins of the lysolipin type. Changes in the responses to lysins have in fact been found in the erythrocytes of anæmic and stored bloods and these are discussed in Part II of the present paper.   It was hoped that a study of the factors affecting the action of lysins on normal cells might throw light on the changes observed in abnormal erythrocytes and some of these factors are considered in Part I.

PART I.

FACTORS AFFECTING THE HÆMOLYSIS OF NORMAL HUMAN ERYTHROCYTES BY LYSINS.

Before considering data more fully, it is necessary to discuss the methods employed.   Lysins are extremely sensitive to physical and chemical agents, and by varying conditions slightly quite different types of response may be secured.   This may, perhaps, account for the enormous literature on the subject and for the seemingly paradoxical results sometimes obtained.   Indeed, possible variations are so numerous that it was found necessary to accept some incompleteness of data and to define the conditions of a limited number of experiments fairly closely.

There are two main methods of measuring the activity of lysins: in the first, the time taken for a dilution of lysin to produce a constant degree of lysis is measured.   A complicated photo-electric layout capable of use with samples immersed in a water-bath is required.   Each test and reading must follow successively, and therefore the time taken for each test must be short and the lysin must be used in relatively concentrated solution.   Hence, results obtained by this method are sometimes different from those observed with other methods where more dilute lysins act for a longer time, since different concentrations of the same lysin may present marked qualitative differences in action.

Ponder [1934 b] measures the time taken for hæmolysis to be complete, but this is undesirable in pathological bloods, for it is possible that a blood containing erythrocytes which are in general less resistant than normal might still contain a few cells which are more resistant.   Comparable to this are those bloods whose cells have a low average size, but which contain a few cells larger than the largest found in normal blood.   Wilbur and Collier [1943] have also considered Ponder's procedure unsatisfactory and have preferred the time taken for a lysin to destroy exactly 50 per cent. of cells.   These authors have found that the transmission of light through a cell suspension of constant strength varies with the size of the erythrocytes being greater with swollen cells in hypotonic solution and less with shrunken cells in hypertonic solution. It seemed to the present writer that this fact excluded the use of a

photo-electric method when examining systems containing cells from macrocytic and microcytic anæmias.

In the second method, the amount of lysis produced by a given solution of lysin acting for a fixed time is measured. The time of exposure is long, the lysin used is dilute, and a number of tests may be put up within a minute or two which for practical purposes may be regarded as simultaneous. The actual readings are independent of cell size and the apparatus needed is simple. The method has been criticised on the grounds that during prolonged action of the lysin in dilute solution erythrocytes settle and are removed from the bulk of lysin, whereas in the first method the experimental period is so short that the lysin acts throughout on a homogeneous suspension. It is possible, however, that this objection is not valid for lysis may well occur in two stages: the first, rapid, consisting of adsorbtion of lysin till cells and fluid are in equilibrium, and the second, slow, consisting of lysis by the adsorbed substance. In support of this view it may be mentioned that cells exposed to lysin and then washed while still unlysed may subsequently show lysis when resuspended in simple saline. However this may be, the same degree of lysis occurs in one hour whether the cells are allowed to settle or kept suspended by gentle mixings (Table I).

TABLE I.—Effects of Lysins on Erythrocytes allowed to Settle or kept Suspended. 1 Hr. at 25°. Phosphate Buffer at pH 7·3.

Erythrocytes.	Per cent. hæmolysis in			
	Bile salts.		Saponin.	
	1/2000.	1/2500.	1/150000.	1/180000.
Settled	42	28	64	50
Suspended . . .	38	28	60	48

Another criticism of this method arises from the possibility that centrifuging, by further damaging cells injured but unhæmolysed by lysin, may add an extraneous increment to the total lysis. But in fact such an increment is not extraneous since it would not be found were the lysin absent and its inclusion does not invalidate the data provided experimental methods are standardised.

In view of the inaccessibility of a photo-electric apparatus and its unsuitability for systems containing cells of varying size, the first method was not investigated.

Method.—The following with appropriate variations was used: citrated blood was centrifuged for 30 minutes at 3000 r.p.m. in tubes

drawn out to sealed graduated and calibrated capillaries holding about 0·1 ml.   The buffy coat was removed, all supernatant plasma washed away, the tube recentrifuged and the volume of erythrocytes accurately read.   The cells were then resuspended with a teat and capillary pipette in fifty volumes of phosphate buffer at pH 7·3 ($NaH_2PO_4.2H_2O$, 2 per cent., 200 ml.; NaOH 2N, 10 ml.; water to 250 ml.).   Washing of cells was avoided because of the risk of damaging the fragile cells of stored and certain pathological bloods.   Intercellular plasma amounts to about 2 per cent. by volume of centrifuged cells and it was thought that qualitative variations in the anti-hæmolytic power of this trace of plasma would not cause significant changes in experimental results.

The cell suspensions were equilibrated in a constant temperature bath (usually at 25°), as were a series of test-tubes containing 1·5 ml. lysin in phosphate buffer.   0·5 ml. suspension was then mixed with the contents of each test-tube.   After exactly one hour the cell suspensions were gently mixed and centrifuged and the amount of hæmolysis in the supernatant fluids compared with a standard in a colorimeter.   It would have been desirable for each experiment to provide complete curves between 0 and 100 per cent., but as a number of different samples had to be examined at the same time, observations had to be restricted to two dilutions of each lysin.

Lysolipin (lysolecithin + lysocephalin) was prepared from egg yolk by the method of King and Dolan [1933].   The bile salts used (British Drug Houses) consisted of a mixture of sodium taurocholate and glycocholate.

Results.—Among the many factors affecting the action of lysins are the following: (1) Relative proportions of erythrocytes and lysins. (2) Effects of tonicity and hence of cell size on lysis.   (3) Temperature. (4) Anions.   (5) pH.   (6) Inhibiting substances.   (7) Mixtures of lysins. Many of these factors have already been investigated by various authors. They are reconsidered here under the special conditions of the present experiments.

(1) *Proportions of Erythrocytes and Lysins.*—The effects of varying the proportions of cells and lysin are shown in Table II.   Here the volume of lysin was kept constant at 2 ml. while the volume of packed cells added to the hæmolytic systems was varied from 0·023 ml. (column 2, top line) to 0·0049 ml. (bottom line).   Column 3 shows the absolute volume of cells lysed when bile salts were present in a final overall content of 1/2000, while column 4 expresses the volume of cells lysed as a percentage of the volume of cells originally present.   Columns 5 and 6, 7 and 8, and 9 and 10 show corresponding data for bile salts 1/2500 and saponin 1/150000 and 1/180000.   In the case of saponin, the absolute amount of cells lysed increases with the erythrocyte content, but the percentage of total cells lysed remains fairly constant, tending to decrease as the cell content falls.   In the case of bile salts, on the other

hand, the absolute amount of cells lysed is roughly constant over a fairly wide range falling only when the suspension used is particularly rich in cells. But the percentage of total cells present which is lysed by bile salts varies inversely with the number of cells in the system. The data suggest that later findings in the case of the anæmias may be complicated by variations in the total surface area of unit volume of cells.

TABLE II.—EFFECTS OF VARYING PROPORTIONS OF ERYTHROCYTES AND LYSINS.
1 HR. AT 25°. pH 7·3.

Hæmolytic system.		Bile salts.				Saponin.			
		1/2000.		1/2500.		1/150000.		1/180000.	
Lysin volume, ml.	Cell volume, ml.	Absolute volume cells lysed, ml.	Total cells lysed, per cent.	Absolute volume cells lysed, ml.	Total cells lysed, per cent.	Absolute volume cells lysed, ml.	Total cells lysed, per cent.	Absolute volume cells lysed, ml.	Total cells lysed, per cent.
2·0	0·023	0·0013	5	0·0010	4·5	0·0164	71	0·0132	57
2·0	0·012	0·0026	22	0·0018	15	0·0080	67	0·0067	56
2·0	0·010	0·0031	31	0·0023	23	0·0070	70	0·0058	58
2·0	0·0083	0·0033	40	0·0026	31	0·0055	66	0·0545	54
2·0	0·0066	0·0032	49	0·0027	41	0·0039	59	0·0031	47
2·0	0·0049	0·0033	67	0·0028	58	0·0029	59	0·0023	47

(2) *Tonicity, Cell Size, and Hæmolysis.*—Equal amounts of cells and lysin were added to each of two phosphate buffers at pH 7·3. , The tonicity of one buffer was such as to cause no change in volume of the erythrocytes added, while the other buffer was hypotonic and caused the cells to swell by 22 per cent. In confirmation of the observations of Wilbur and Collier [1943] it was found that lysis by saponin was inhibited by cell swelling, while lysis by lysolecithin was increased, and a similar increased lysis was observed in the case of bile salts, oleate, and streptolysin O (Table III). It is, of course, not possible to decide how far the altered response to lytic action is due to the effects of varying degrees of swelling on the cell surface and how far to the varying salt content of the external medium.

(3) *Temperature Coefficient of Lytic Action.*—Table IV shows that with saponin lysis increases sharply between 4° and 25°, after which it remains more or less constant. With bile salts lysis falls sharply between 4° and 18°, reaches a minimum between 20° and 25°, and then rises sharply again, while with oleate lysis increases slowly between 20° and 25° and then sharply up to 36°. It is this marked effect of

TABLE III.—ACTION OF LYSINS ON SWOLLEN AND UNSWOLLEN CELLS.
1 HR. AT 25°. pH 6·3.

External solution type.	External solution composition.			Cell volume.		Per cent. hæmolysis in			
	P. m.gm. per cent.	Cl. m.gm. per cent.	Volume, ml.	Initial, ml.	Final ml.	Saponin, 1/120000.	Lysolipin, 1/100000.	Bile salts, 1/2000.	Oleate, 1/40000.
Constant phosphate . .	240	128	2·0	0·01	0·0121	76	36	42	62
Constant volume	360	128	2·0	0·01	0·01	96	28	26	44

temperature on lysis which makes it so important to conduct experiments in a thermostatically controlled water-bath. Indeed, the wide temperature spacings in Table IV are due to the limited number of constant temperature water-baths available.

TABLE IV.—EFFECTS OF TEMPERATURE ON LYTIC ACTION IN PHOSPHATE
BUFFERS. 1 HR. AT pH 7·3.

Temperature.	Per cent. hæmolysis in					
	Bile salts.		Saponin.		Oleate.	
	1/1750.	1/2000.	1/150000.	1/180000.	1/45000.	1/55000.
4°	95	83	6	4	12	8
18°	59	44	..	..	..	..
20°	52	38	35	26	23	13
25°	59	37	58	50	25	16
36°	100	100	56	48	100	70

(4) *Effects of Ions on Hæmolysis.*—This was investigated in the case of phosphate-chloride mixtures only: the addition of chloride within the range examined inhibited lysis (Table V).

(5) *pH and Lysis.*—The action of lysins is greatly affected by changes in reaction and the effects of pH are themselves modified by quantitative and qualitative changes in the salts present. In the present experiments, 1 part of a two per cent. cell suspension in saline (0·85 per cent.) was added to three parts of phosphate buffer containing the lysin. pH was measured colorimetrically on duplicate systems centrifuged before the cells had had time to lyse. The method is approximate, but on the other hand, owing to heavy buffering, shift in pH due to liberation of hæmoglobin will be much less than in the more lightly buffered systems of Bodansky [1929] and Gordon [1932–33].

TABLE V.—EFFECTS OF VARYING PROPORTIONS OF PHOSPHATE AND CHLORIDE ON LYSIS.   1 HR. AT 23°.   pH 7·3.

Isotonic phosphate parts.	Isotonic chloride parts.	Per cent. hæmolysis in			
		Bile salts.		Saponin.	
		1/1600.	1/2000.	1/150000.	1/180000.
4	0	81	63	59	50
3	1	45	34	52	40
2	2	33	25	41	33

The first series of experiments were carried out with a series of buffers of varying pH and constant phosphate content (figs. 1–9). Varying amounts of caustic soda were added to equal amounts of acid sodium phosphate, the appropriate amount of lysin added and each sample made up to the same volume with water. All contained 240 mg. per cent. inorganic phosphorus. Acid buffers caused erythrocytes to swell while alkaline buffers caused shrinkage; the sample at pH 7·3 being isotonic. It follows that in acid ranges, in accordance with the observations of Wilbur and Collier [1943] cell swelling due to pH (as distinct from the effects of pH itself) will inhibit the lytic effects of saponin, while those of other lysins will be enhanced. Accordingly, a second series of buffers was prepared in the same way as the first, except that less water was added in the acid range and more in the alkaline range, the quantity being so adjusted that when erythrocytes were added no change of cell volume occurred whatever the pH of the buffer sample used. The amount of inorganic phosphorus in these constant volume buffers is shown in fig. 2. The effects of pH on lytic action with these constant volume buffers are shown by the broken line in figs. 4–9.

It follows that erythrocytes will show no change of volume at pH 7·3 with either series of buffers, but cells in the first series will swell progressively as pH falls, while the volume of cells in the second series will remain unaltered. Hence any effect of swelling on lysis should be progressively more marked as pH falls, and reference to figs 4–9 shows progressive inhibition in the case of saponin and progressive enhancement in the case of lysolipin, bile salts, oleate and streptolysin O. It will be further understood that it is not possible to dissociate the direct effects of pH on lysis from secondary effects due to associated alterations in the ionic composition of the buffers used.

In addition to these two series of experiments with phosphate buffers, a third series of pH curves was obtained using diluted citrated blood: whole blood, 4 parts; citrate 1 part, and saline 35 parts; pH was adjusted with HCl or NaOH (figs. 4–7).

CELL VOLUME CHANGES & pH.

x—x CONSTANT PHOSPHATE VARIABLE VOLUME

+---+ VARIABLE PHOSPHATE CONSTANT VOLUME

% VOLUME

pH

FIG. 1.

EXTERNAL PHOSPHORUS TO GIVE NO CHANGE IN CELL VOLUME WITH pH. (Cl = 128 MG %)

EXTERNAL INORGANIC PHOSPHORUS MG %

pH

FIG. 2.

HAEMOLYSIS BY STORAGE

BLOOD 80% IN CITRATE AT 14 DAYS. ●—●

BLOOD 3% IN PHOSPHATE AT 35 DAYS. x—x

% HAEMOLYSIS

pH

FIG. 3.

HAEMOLYSIS BY LYSOLIPIN.

x—x CELLS IN VARIABLE VOLUME CONSTANT PHOSPHATE BUFFER

+---+ CELLS IN CONSTANT VOLUME VARIABLE PHOSPHATE BUFFER

●—● BLOOD IN CITRATE SALINE

% HAEMOLYSIS

1/6000   1/100000

pH

FIG. 4.

HAEMOLYSIS BY BILE SALTS.

x—x CELLS IN VARIABLE VOLUME CONSTANT PHOSPHATE BUFFER

+---+ CELLS IN CONSTANT VOLUME VARIABLE PHOSPHATE BUFFER

●—● BLOOD IN CITRATE SALINE

% HAEMOLYSIS

1/220   1/2000

pH

FIG. 5.

HAEMOLYSIS BY SAPONIN.

   ×——× CELLS IN VARIABLE VOLUME
        CONSTANT PHOSPHATE BUFFER

   +----+ CELLS IN CONSTANT VOLUME
        VARIABLE PHOSPHATE BUFFER

   ●——● BLOOD IN CITRATE SALINE

HAEMOLYSIS BY OLEATE.

   ×——× CELLS IN VARIABLE VOLUME
        CONSTANT PHOSPHATE BUFFER

   +----+ CELLS IN CONSTANT VOLUME
        VARIABLE PHOSPHATE BUFFER

   ●——● BLOOD IN CITRATE SALINE
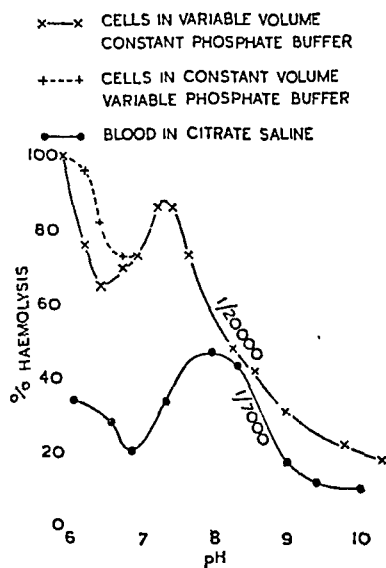
FIG. 6.
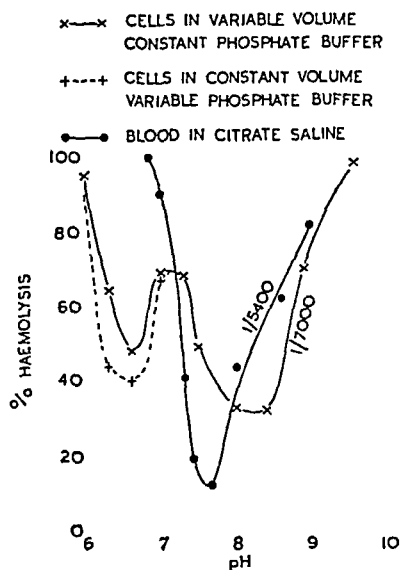
FIG. 7.

HAEMOLYSIS IN PALMITATE.

×—×CELLS IN VARIABLE VOLUME
CONSTANT PHOSPHATE
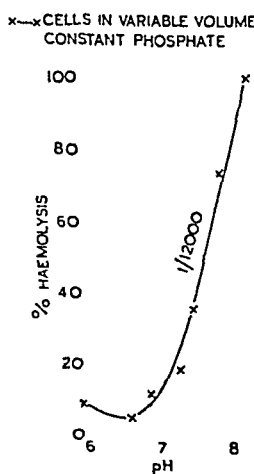
HAEMOLYSIS BY STREPTOLYSIN.O

×—×CELLS IN VARIABLE VOLUME
CONSTANT PHOSPHATE.
+---- CELLS IN CONSTANT VOLUME
VARIABLE PHOSPHATE
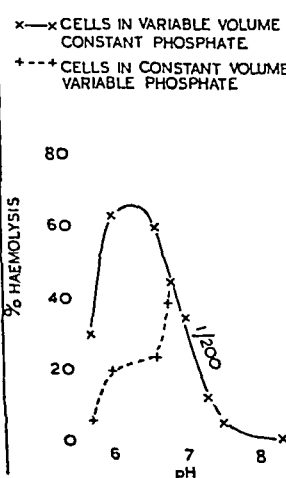
INHIBITION OF SAPONIN AND
REINFORCEMENT OF BILE SALTS IN
MIXTURES WITH LYSOLIPIN.

×—× SAPONIN 1/150000 + LYSOLIPIN

●—● BILE SALTS 1/2500 + LYSOLIPIN

+--+ LYSOLIPIN ALONE

FIG. 8.

FIG. 9.
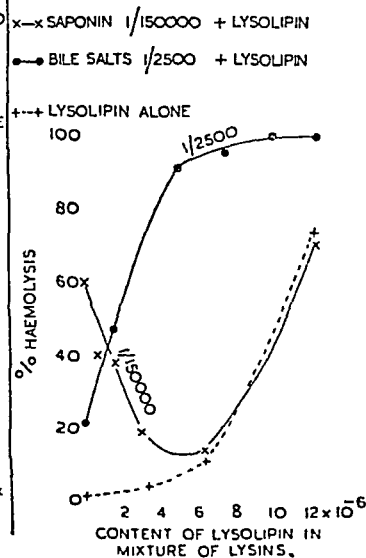
FIG. 10.

With regard to phosphate systems, it may be said that all curves with the exception of lysolipin and streptolysin O show minimum lysis between pH 6·3 and 6·6 with increased lysis on either side. With palmitate (fig. 8) and lysis due to storage (fig. 3), at pH greater than 6·6 lysis increases until it becomes complete, while with bile salts (fig. 5), saponin (fig. 6), and oleate (fig. 7), a peak is reached between pH 7 and 7·4 after which lysis again declines. In the case of oleate, a final increase begins at pH 8, while with bile salts and saponin lysis remains low until the respective pH 9·5 and 10, when again it increases rapidly—possibly because the lytic effect of the alkali is added to that of the lysin itself. The curve for streptolysin O is simple, showing a maximum between pH 6 and 7; the effects of salt content acting directly or through volume changes induced is very marked (fig. 9).

The curves differ somewhat from those of other workers, partly, perhaps, because of the different technique used. Thus in the present series, blood was citrated: as citrate penetrates erythrocytes with extreme slowness [Maizels, 1943] there will only be a faint trace of this anion in the hæmolytic system, derived from intercellular plasma in the unwashed cells. Apart from this, the systems were heavily buffered with phosphate, a feature present in Wilbur and Collier's experiments, but not in those of Bodansky [1929] or of Gordon [1932–33]. The lysolipin curve is very like that of Wilbur and Collier. The bile salt curve between pH 6 and 10 differs from that of Gordon in showing a decrease of resistance between pH 7 and 9, and this may be due to our use of mixed salts instead of pure taurocholate or to closer pH spacings and to more clear cut effects in the presence of phosphate buffers: in Gordon's work, the pH spacings were 6, 7·1, 8·1, and 9. The saponin curve differs from that of Bodansky [1929] in having a minimum between pH 6 and 8, while his present a plateau. This may be due to the presence of phosphate in our preparations, or to the fact that there are different kinds of saponins with individual peculiarities [Norris, 1939] or to a failure of Bodansky to record the effects on hæmolysis of pH between 6 and 8. The differences between present and earlier data obtained by the writer probably arise from different temperatures of observation, – 25 as compared with 16°.

The curves for citrated whole blood in saline differ from those of phosphate systems in being simpler. The citrate-saline systems are much less well buffered between pH 6 and 8, so that there will be a "sliding" pH for each individual observation, with a general smoothing out of each curve as a whole. Thus bile salts and oleate present a single minimum at about pH 7·5, with increased lysis above and below this point. But the saponin curve remains complex with a secondary maximum at pH 8 instead of pH 7·3. In the case of ordinary citrated blood (with glucose to 0·5 per cent.) undiluted with saline and stored for fourteen days at 2°, lysis was least between pH 6·4 and 7, while a 3 per

cent. suspension of whole blood in phosphate buffers showed a single minimum at pH 6·8 (fig. 3).

SUBSTANCES INHIBITING LYSIS.

These include sugars, polysaccharides, cholesterol, lecithin, and proteins, and in addition the group soaps, bile salts, and lysolipin which inhibit saponin and streptolysin O.

Sugars.—It is to be expected that the action of sugars on lysins will be complex and difficult to analyse.   In the absence of lysin, addition of sugar to saline raises tonicity and causes cells to shrink.   In order to keep tonicity and cell size constant it will be necessary to decrease the salt content as sugar is added and this alone will influence the response to lysin.   In addition, if the sugar content of the suspending solution is high and the salt content low, salts in the interior of the cells hydrolyse with an escape of traces of anion sufficient to alter the external pH to between 5 and 6.   Later cations leak out and the pH again rises [Maizels, 1935] while the cell shrinks.   This will be counteracted by an inflow of sugar and water into the cell if the substance added is glucose, but not if it be sucrose.   It is therefore doubtful if experiments are quite valid when solutions are used which are rich in sugar, poor in salts, and where changes in cell volume and pH are unrecorded.   Such is the case in some results of Yeager [1929] which seem to show marked inhibition of saponin and sodium taurocholate by sucrose.   According to Tsai and Lee [1941] glucose inhibits the rapid lysis of strong saponin solutions and accelerates the slow lysis of weak saponin.   With lysins in phosphate solutions, where shifts of pH are minimised and only moderate amounts of sugar are added inhibition was slight (Table VI).   On the other hand, glucose strongly inhibits the natural hæmolysis of storage while sucrose is without effect.   Probably, the action during storage is exerted within the cell on the metabolic cycle and not on any lysin acting on the surface [Maizels, 1943].

TABLE VI.—EFFECTS OF SUGARS IN PHOSPHATE BUFFERS ON LYSIS.
1 HR. AT 23°.   pH 7·3.

Inhibitor.	Per cent. hæmolysis in			
	Lysolipin 1/100000.	Saponin 1/100000.	Oleate 1/40000.	Bile salts 1/2000.
None. . . .	90	96	62	60
Glucose 1 per cent. .	86	88	42	62
Sucrose 1 per cent. .	88	98	50	52

Polysaccharides.—Maizels and Whittaker [1940] showed that dextrin and starch inhibited hæmolysis in stored blood; inulin had the same

effect. Dextrin also inhibits all lysins examined except streptolysin O, the effects being quite marked even in strong mixtures of cells and lysin. Dextrin used was slightly acid in reaction and was adjusted to pH 7·3 before use (Table VII).

TABLE VII.—INHIBITION OF LYSINS BY DEXTRIN IN PHOSPHATE BUFFERS.
1 HR. AT 23°. pH 7·3.

Lysin.	Cell suspension.	Per cent. hæmolysis in the presence of inhibitor—dextrin per cent.			
		2.	1.	0·5.	0.
Saponin 1/100000 . .	1/200	38	71	86	92
Bile salts 1/2000 . .	1/200	0	17	32	97
Oleate 1/40000 . .	1/200	0	0	10	71
Streptolysin O 1/200 .	1/200	42	52	52	50
Lysolipin 1/100000 .	1/200	0	0	5	98
Lysolipin 1/4000 .	1/5	51	60	62	64

Cholesterol.—The inhibition by cholesterol of lysis by saponin was described by Ransom [1901] and Stocks [1919–20], while Hewitt and Todd [1939] showed that cholesterol would inhibit streptolysin O but not S. In phosphate buffers it was found to inhibit all lysins tested except complement. It was also without effect on the natural lysis of storage. A very fine suspension of cholesterol was prepared by dissolving 0·4 g. in 5 ml. hot alcohol, adding 20 ml. of a solution of trieth-anolamine (0·2 per cent.) at 60° and dialysing for two weeks against distilled water.

TABLE VIII.—EFFECTS OF CHOLESTEROL ON LYSIS. 1 HR. AT 25°. pH 7·3.

Lysin.	Per cent. hæmolysis in the presence of inhibitor cholesterol mg. per cent.				
	12.	6.	3.	1·5.	0.
Saponin 1/150000 . .	0	28	30	36	46
Lysolipin 1/88000 . .	0	0	0	34	96
Streptolysin O 1/80 . .	0	0	2	..	97

Similar inhibitions of bile salts and oleate were observed using cholesterol emulsified with dried human serum in water, though this preparation was less efficient than that prepared by the method above.

Phospholipin.—The preparation used consisted of a mixture of lecithin and cephalin prepared from egg yolk. Unlike commercial lecithin, which contains products of decomposition and is hæmolytic, this preparation was cream coloured and non-hæmolytic. As mentioned by Ponder [1934] phospholipin inhibits bile salts; it also inhibited oleate and lysolipin. It appeared to inhibit saponin, but this may have been due to traces of impurity. Streptolysin O was also slightly inhibited, and it is likely that the marked inhibition of streptolysin O and S observed by Hewitt and Todd [1939] was due to impurities in the commercial preparation they used (Table IX).

TABLE IX.—INHIBITION OF LYSINS BY PHOSPHOLIPIN. 1 HR. AT 23°.
PHOSPHATE BUFFER, pH 7·3.

Lysin.	Per cent. hæmolysis in the presence of inhibitor phospholipin.						
	1/4000.	1/8000.	1/16000.	1/32000.	1/64000.	1/128000.	0.
Saponin, 150000 .	0	0	12	30	36	42	54
Bile salts, 1/1800 .	0	8	..	56	66	75	81
Oleate, 1/40000 .	0	0	0	10	14	39	84
Streptolysin O, 1/96	87	94	98	96	94	96	96
Lysolipin, 1/80000 .	14	20	31	41	49	53	56

With regard to the effects of impurities in the phospholipin: it was found that a fresh cream-coloured preparation inhibited saponin much less than an older ambler-coloured one, while both inhibited bile salts equally well (Table X).

TABLE X.—EFFECT OF PURITY OF PHOSPHOLIPIN ON THE INHIBITION OF LYSIS BY SAPONIN AND BILE SALTS. 1 HR. AT 25°. PHOSPHATE BUFFER, pH 7·3.

Lysin.	Inhibitor.	Per cent. hæmolysis in the presence of inhibitor phospholipin.					
		1/40000.	1/80000.	1/160000.	1/320000.	1/640000.	0.
Saponin 1/150000	Phospholipin, more pure	62	66	70	70	72	70
Saponin 1/150000	,, less ,,	32	44	48	52	66	68
Bile salts 1/2000	,, more ,,	17	29	31	39	40	41
Bile salts 1/2000	,, less ,,	17	29	35	41	42	42

Inhibition of One Lysin by Another.—Table XI shows the effects of mixing lysolipin (column 1) with various other lysins. The first horizontal line shows the effects of mixtures containing relatively high

contents of lysolipin and here hæmolysis is marked. Subsequent horizontal lines show the effects of a progressive decrease in the content of lysolipin. In the case of all lysin mixtures, there is at first a corresponding decrease in lysis (lines 2, 3, 4, and 5) which is most marked with saponin and streptolysin O. With further decrease in the amount of lysolipin added (lines 6, 7, and 8), lysis by saponin (columns 2, 3, 4, and 5) and by streptolysin O (column 6) again increases, showing that lysolipin inhibits these two lysins when present in small amounts, but not when the amount present is very small indeed. In the case of bile salts (columns 7, 8, 9, and 10) and of oleate (column 11) lysolipin exerts no inhibition, the total amount of hæmolysis decreasing throughout as the content of lysolipin falls. These findings are also shown in fig. 10.

Bile salts (Table XII) also inhibits saponin and streptolysin O, but not palmitate, while palmitate also inhibits saponin and streptolysin O (Table XIV). When saponin and streptolysin O act simultaneously on erythrocytes, their effects seem to be additive except in the case of dilute

TABLE XI.—PERCENTAGE OF HÆMOLYSIS DUE TO THE SIMULTANEOUS ACTION OF LYSOLIPIN WITH OTHER LYSINS. 1 HR. AT 25°. PHOSPHATE BUFFER, pH 7·3.

Lysolipin with	Saponin.				Strepto-lysin O.	Bile salts.				Oleate.
	1/100000.	1/150000.	1/180000.	0.	1/96.	1/1850.	1/2500.	1/7500.	0.	1/128000.
1/80000 .	72	69	72	78	74	100	100	88	80	100
1/100000	..	..	..	..	..	100	100	67	46	..
1/150000	30	14	10	12	12	100	95	38	14	..
1/200000	..	..	..	..	4	100	91	12	4	..
1/320000	63	19	18	4	4	..	..	..	..	42
1/640000	66	37	32	2	53	86	46	2	2	..
1/1280000	84	40	38	2	94	..	..	..	..	..
0 .	98	60	45	1	94	68	31	1	1	24

TABLE XII.—PERCENTAGE OF HÆMOLYSIS DUE TO THE SIMULTANEOUS ACTION OF BILE SALTS WITH OTHER LYSINS. 1 HR. AT 25°. PHOSPHATE BUFFER. pH 7·3.

Bile salts with	Saponin				Strepto-lysin O.	Palmitate		
	1/100000.	1/150000.	1/200000.	0.	1/120.	1/12000.	1/24000.	0.
1/1600 . .	82	80	82	81	83	100	100	89
1/2000 . .	55	52	47	52	..	100	75	50
1/4000 . .	14	13	12	12	10	..	..	..
1/8000 . .	15	5	3	3	47	..	..	..
1/16000 . .	40	31	14	44	..	..	..	..
0 . .	100	68	45	2	54	44	10	0

solutions of streptolysin O. Table XV suggests that this inhibition is due not to the lysin itself but to some other substance present with it in solution.

TABLE XIII.—PERCENTAGE OF HÆMOLYSIS DUE TO THE SIMULTANEOUS ACTION OF PALMITATE WITH OTHER LYSINS. 1 HR. AT 25°. PHOSPHATE BUFFER, pH 7·3.

Palmitate with	Saponin.		Streptolysin O.
	1/150000.	0.	1/96.
1/12000	56	44	..
1/24000	20	10	21
0	72	0	88

TABLE XIV.—PERCENTAGE OF HÆMOLYSIS DUE TO THE SIMULTANEOUS ACTION OF SAPONIN WITH STREPTOLYSIN O. 1 HR. AT 25°. PHOSPHATE BUFFER, pH 7·3.

Saponin with	Streptolysin O.				
	1/160.	1/230.	1/320.	1/1600.	0.
1/150000 . .	93	56	30	23	40
1/215000 . .	95	47	20	6	15
1/300000 . .	89	48	13	6	11
1/750000 . .	81	36	9	4	2
0 . .	58	19	4	2	0

It has been seen that lysolipin and bile salts inhibit saponin when the lysins act simultaneously, this inhibition is also evident when the lysins act successively: erythrocytes were exposed to a dilute solution of lysolipin or bile salts, immediately centrifuged in capillary-ended centrifuge tubes, the supernatant fluid carefully washed away and the cells resuspended in saponin solution. After one hour, lysis was measured in this tube and in a control where the preliminary exposure was to simple phosphate buffer without added lysin, followed by exposure to saponin. It was found that saponin caused less lysis of cells which had first been exposed to lysolecithin in subhæmolytic concentration. So, too, erythrocytes exposed to phosphate and then to saponin were more hæmolysed than cells treated with a very dilute solution of bile salts in phosphate followed by saponin solution (Table XV).

TABLE XV.—INHIBITION OF LYSIS BY SAPONIN THROUGH PRELIMINARY EXPOSURE TO LYSOLIPIN, ETC.

Preliminary brief exposure to 2 ml. of	Followed by exposure to 2 ml. saponin, 1 hr. at 25°, pH 7·3. Per cent. hæmolysis in saponin	
	1/150000.	1/180000.
Phosphate control—no lysin .	81	55
Lysolipin, 1/180000 in phosphate	44	20
Bile salts, 1/2000 . . .	64	32

From all the foregoing it may be concluded that when lysolipin, bile salts, or soaps are adsorbed on erythrocytes, the action of saponin is inhibited and this inhibition may be used as a test for the presence of adsorbed lysin of the lysolipin type. It may also be shown that the application of lysolecithin or bile salts after saponin will inhibit lysis. Thus, if erythrocytes are exposed to a strong solution of saponin which is immediately replaced by simple phosphate buffer, sufficient saponin will have been adsorbed to cause subsequent lysis. But if the buffer contain a suitable amount of lysolipin or bile salts and is added soon enough after the preliminary exposure to saponin, lysis by saponin may be inhibited (Table XVI).

TABLE XVI.—INHIBITION OF LYSIS BY SAPONIN ON THE SUBSEQUENT ADDITION OF LYSOLIPIN, ETC. 1 HR. AT 25°. pH 7·3.

(Preliminary momentary exposure of erythrocytes to saponin solution (1/40000) followed by centrifuging and replacement of saponin by the second solution at intervals of 1 to 15 minutes. Hæmolysis observed after second solution has acted for 1 hour.)

Interval between exposure to saponin and addition of the second solution, mins.	Per cent. hæmolysis with second solution.				
	Simple phosphate.	Bile salts, 1/2500.	Bile salts, 1/5000.	Lysolipin, 1/120000.	Lysolipin, 1/240000.
1	72	39	54	33	33
5	70	38	..	44	29
15	76	56	..	70	54

Proteins.—The inhibitory effects of plasma proteins is well known [Bayer, 1907; v. Lieberman, 1907; Ponder, 1934 b]. Their effects under the present experimental conditions are shown in Table XVII. The

first line shows lysis in washed cells, the second in unwashed cells containing 2·1 per cent. plasma by volume; the third.system (line 3) has sufficient plasma added to double the plasma in system 2, while the fourth has triple this amount.

TABLE XVII.—EFFECTS OF PLASMA ON LYSIS BY BILE SALTS AND SAPONIN.
1 HR. AT 25°. pH 7·3.

Cells ml.	Plasma ml.	Per cent. hæmolysis in	
		Bile salts 1/2000.	Saponin 1/180000.
0·01	0	48	57
0·01	0·0002	44	50
0·01	0·0004	42	45
0·01	0·0006	40	41

DISCUSSION.

Little is known about the mode of action of hæmolysins which is often paradoxical and complex. It would seem most likely that the erythrocyte membrane consists of a complex film of protein and lipin, including lecithin and cholesterol. Lysins must be assumed to act by destroying the adhesion of such surface elements. Norris [1939] suggests that saponin attacks the lipoid portions, while Plattner and Hintner [1930] ascribe a similar action to bile salts and oleate. Schulman and Rideal [1937] have shown that soaps will displace protein from a surface film and form a monolayer of fatty acid. They show that saponin combines with cholesterol and that fatty acids and taurocholate also combine with cholesterol and have the additional property of dispersing monolayers of protein. They suggest that this may explain why erythrocytes treated with saponin leave well-formed "ghosts", while those treated with fatty acids are completely disintegrated. Cholesterol in the erythrocyte membrane may have the effect of stabilising proteins and lecithin. Thus Leathes [1923] showed that lecithin alone gave a loose expanded film which in the presence of cholesterol became condensed, while Hughes found [1935] that snake venom rapidly attacked the expanded film of lecithin, but was inhibited by cholesterol which condenses the film and prevents access of the venom. It will thus be apparent that factors affecting lysis may act on the constituents of the cell surface in relation to their isoelectric points, their orientation at the cell surface, their capacity to form micelles, and their chemical affinities, and these same factors may act similarly on the lysin itself.

Tonicity.—In so far as this alters the shape of the cell, it will affect

the curvature of the surface. Nevertheless, when the dimensions of the surface molecules are considered in relation to the size of the cell as a whole, it will be obvious that the cell surface will always be relatively flat and the effects of tonicity on the orientation of surface molecules will be quite minute and will hardly affect their accessibility to lysins. It is probable that the effects of tonicity on lysis observed by Wilbur and Collier [1943] and by the present writer are best explained by the work of Davson and Danielli [1938]. These writers have shown that exposure of erythrocytes to subhæmolytic concentrations of many lysins (saponin is an exception) increases the permeability to salts so that lysis by these agents is due partly to disintegration of the cell envelope and partly to osmotic swelling resulting from increased permeability. It is therefore to be expected that when erythrocytes are exposed to lysins in hypotonic solution the osmotic factor will be augmented and lysis will occur more readily. Saponin, on the other hand, causes lysis without first increasing permeability to salts [Davson and Danielli, 1938], and hypotonicity does not increase its lytic action. Salt permeability effects, however, cannot explain the actual decreased susceptibility to saponin observed in hypotonic solutions, and this must be ascribed to those changes in salt content which are inseparable from changes in tonicity.

Proportions of Erythrocytes and Hæmolysin.—In the case of saponin (Table II) it was found that within a fairly wide range, increase of the number of cells in a system caused a corresponding increase in the absolute amount of lysis, the proportion of cells lysed relative to the whole remaining fairly constant. Since the amount of lysin adsorbed depends on the concentration outside the cells and since doubling the number of cells almost doubles the lysis, it follows that very little of the saponin available is actually adsorbed, for otherwise the content of saponin in the bulk phase would fall as the number of cells increased and so the percentage of cells lysed would decrease. With bile salts (and also lysolipin), on the other hand, by the time equilibrium is reached with the cells, a fairly large proportion of the lysin available will have been adsorbed, for the percentage of cells lysed falls rapidly as the total number of cells available is increased. The findings suggest that results in the case of the anæmias may be complex owing to quantitative variations in the extent of cell surface and qualitative differences in composition.

Temperature Coefficients, Ions and pH.—All these may affect the orientation of surface molecules, the aggregation of lysin micellæ, the rate of adsorbtion and the amount of a substance adsorbed, and their respective actions on biological processes in general are well known but ill understood. This is especially true of hæmolysis where the resultant of all these individual effects has not been susceptible of analysis. In the case of hydrogen ion concentration Bodansky [1929] and Ponder

[1934 b] suggest that its chief effect is on the erythrocyte and not on the lysin. It is possibly this constant cell factor which leads to most lysins having a minimum effect between pH 6·2 and 6·6, the exceptions being streptolysin O which has a maximum in this range, and lysolipin which lyses least at about pH 8.

Inhibitory Substances.—It has already been noted that the addition of sugars to hæmolytic systems involves alterations in tonicity or ionic composition, together with changes in pH and cell volume. How far such undetermined changes are responsible for the inhibition described by various authors and how far sugar itself is directly responsible is not known; under present experimental conditions, inhibition by sugars was slight. Polysaccharides presumably inhibit lysis by adsorbing at the cell surface and so preventing access of lysin or else by forming complexes with lysin in the bulk phase.

It is probable that cholesterol has the twofold effect of stabilising the cell surface and of combining with lysin in the extra-cellular medium. But if more than enough lysin be present to combine with cholesterol outside and on the cell, the excess will displace normal constituents from the cell surface and so cause lysis. This view is supported by Schulman and Rideal's [1937] observations on the formation of complexes between cholesterol and lysins in surface films. Such complexes may be demonstrated by shaking finely dispersed cholesterol with lysins or proteins. If the watery material is now shaken with ether it "clears" much less than a simple watery suspension of cholesterol and the suspended material adsorbs at the air-water interface. It is probable that the cholesterol complex consists of a core of cholesterol surrounded by an ether-resistant layer of lysin or protein comparable to the lipoid-protein complexes of plasma and serum described by McFarlane and Kekwick [1942]. In these complexes the protein (β globulin) forms an external layer protecting the lipin from the action of ether and imparting the electrophoretic mobility of β globulin to the whole particle. It is likely that similar complexes formed with lysins account for the inhibitory action of lecithin and plasma proteins. According to Tsai and Lee [1941] the inhibitory action of plasma is due not so much to the proteins as to the cholesterol, in which case it is to be expected that the free cholesterol alone will be active and not the combined, this view is supported by the work of Hewitt and Todd [1939] on strepto-lysin O and of Collier and Wilbur [1944] in the case of lysolecithin. These observations may be correlated with the observation of Schulman and Rideal [1937] that digitonin associates strongly with sterol –OH and very weakly with sterol ester.

The inhibition of one lysin by another also probably depends on complex formation—a view held by Ponder [1934 a]—and if so, the lytic portion of the saponin molecule must be attached to the non-lytic part of the lysolipin, oleate, palmitate, or bile salt molecule, since, as we have

seen, the former is inhibited by the latter, but the latter is not inhibited by saponin.

SUMMARY.

The action of lysins is greatly affected by temperature, pH, salt content, and the physical state of the cell surface. Increase in cell volume is associated with increased susceptibility to bile salts, lysolipin and soaps, and with decreased susceptibility to saponin.

Most lysins are inhibited by dextrin and cholesterol. Lecithin inhibits bile salts, soaps, and lysolipin. Its action on saponin is slight and it does not inhibit streptolysin O.

If in a sample of blood, erythrocytes show increased lysis by bile salts, soaps, and lysolipin, the possibility exists that some agent had already caused the cells to swell or else that a substance having some of the properties of soaps or lysolipin had appeared spontaneously in the blood.

PART II.

EFFECT OF LYSINS ON THE ERYTHROCYTES OF STORED AND ANÆMIC BLOOD.

It has long been known that certain chemicals and bacterial toxins will cause anæmia and that hæmolysins of the immune body type cause paroxysmal and nocturnal hæmoglobinuria. A recent paper by Foy and Kondi [1943] shows that in blackwater fever, after transfusion, donor's cells are as rapidly destroyed as those of the recipient and that both develop an increased susceptibility to lysolecithin acting *in vitro*. The authors suggest that this affords evidence for the presence of a lysin in the circulating blood. Again, Brown, Hayward, Powell, and Witts [1944] have found that when stored blood is transfused to normals the rate of destruction of donor's erythrocytes is almost linear. This may be considered as the curve for the destruction of erythrocytes by normal physiological agencies. Similar curves are obtained in many anæmias, including pernicious anæmia and congenital familial acholuric jaundice, and it must be presumed that in such cases the hæmolytic mechanism acts normally and that the excessive destruction of recipient's cells is due to inherent defects in his erythrocytes and not in the hæmolytic processes. In acquired non-familial acholuric jaundice, and in certain other anæmias, on the other hand, the destruction of donor cells is very rapid in the first few days after transfusion, after which the rate approximates to normal. It is thought that this initial rapid destruction indicates the presence of an abnormal or pathological hæmolytic factor capable of acting on normal or abnormal cells and presumably of cellular or humoral origin. Evidence that other anæmias may be due to the action of lysins rests on a much less firm foundation. Bergenheim

and Fahraeus [1936] and Fahraeus [1939] have shown that if drawn blood is incubated at 37° without agitation, a hæmolytic substance appears in the serum which is closely related to lysolecithin. Its appearance is delayed or prevented by agitation. These writers were of the opinion that the circulation of the spleen, which according to Knisely [1936] was largely cut off from the main blood flow, would permit the development of their lysin in a way not possible in other parts of the body where the circulation was more active. The argument, however, loses force if the concept of splenic stasis is incorrect, which according to Whipple [1941] is the case. On the other hand, the spleen does seem to have some association with the production of lysolecithin, for it has shown that a lytic substance having the solubilities of lysolecithin can in fact be isolated from incubated sera, that unincubated blood from the splenic vein contains more lysolecithin than blood from the splenic artery [Singer, 1941], and that after splenectomy the lysolecithin content of peripheral blood is less than normal [Singer, Miller, and Dameshek, 1941]. All this might suggest the possibility of hæmolysis resulting from the action of a lysin in the spleen. But Singer himself [1941] adduces evidence to the contrary in that the erythrocytes of blood from the splenic vein are not more susceptible to the action of lysolecithin than are the cells from the artery. Singer concludes that lysolecithin is probably not concerned in physiological or pathological hæmolysis, a view which is endorsed in a critical and original study by Lloyd [1941]. Further support for the view that the defect in acholuric family jaundice is primarily in the erythrocyte is afforded by chemical analysis. Maizels [1936] has shown that the acholuric cell has a low content of water, potassium and total base, and a high content of hæmoglobin. It is conceivable that a lysin in subhæmolytic amounts might increase cell permeability, but in this case water would increase, the concentration of base would be normal, while potassium and hæmoglobin would fall. The abnormalities actually found suggest that the special peculiarities appear at an early stage in cell development.

In the case of blood stored at 2° the work of Fahraeus [1939] might lead one to expect that lysolecithin derived from lecithin by enzyme or other action was the active agent in natural spontaneous hæmolysis. The present writer knows of no direct evidence in support of this possibility and has failed to isolate the substance himself.

It seemed to the writer that the problem might be examined afresh in the light of observations made in Part I, that when erythrocytes have been exposed to subhæmolytic doses of lysins belonging to the bile salts, lysolecithin, soap group their resistance on exposure to the same group is decreased, while resistance to saponin is increased. Experiments have in fact shown that in acholuric jaundice and pernicious anæmia, erythrocytes are less resistant to bile salts and lysolecithin, but they are not more resistant to saponin. In the case of stored blood, hæmolysis

by bile salts is increased while lysis by saponin is decreased, but the findings have no correlation at all with the natural hæmolysis observed. It is thought, therefore, that although one or other of the lysolecithin-soap series increases during storage, it must play a very minor part in natural hæmolysis.

Action of Lysins on Stored Blood.—These experiments were carried out in a way similar to those described on p. 189, but glucose (to 0·5 per cent.) and sodium fluoride (to 0·02 per cent.) were added to the phosphate buffer. The temperature was 23°. It was ultimately felt that no special purpose was served by the addition of fluoride. Since erythrocytes swell during storage, it would not suffice to prepare a standard suspension of 0·1 ml. erythrocytes in 5 ml. buffer, for 0·1 ml. stored cells might only equal 0·075 ml. of the same cells when fresh. Therefore, in this series of experiments all cell suspensions were standardised to give 0·15 g. per cent. hæmoglobin in the final mixtures of cells and lysins. Two dilutions each of bile salts and saponin were used to give final values respectively of 1/2500 and 1/3500; 1/80000 and 1/120000 on mixture with the cell suspensions. Bile salts (B.D.H.) were used rather than lysolipin since the preparation is more easily obtainable and mixes freely with water, whereas lysolipin involves a tedious preparation, is not very stable, is difficult to emulsify with buffer and adsorbs readily on glass [Wilbur and Collier, 1943].

Results obtained on fresh blood were rather more variable than expected and for this reason average figures and standard deviations are shown in Table XVIII. The figures represent percentages of hæmolysis after a blank value has been subtracted equal to hæmolysis observed on adding 0·5 ml. of the corresponding cell suspension to 1·5 ml. phosphate buffer without lysin. The blank equalled 0 up to two weeks of storage, while at four weeks it was 1–2 per cent. for cells stored with acid glucose citrate, 2–3 per cent. for glucose citrate diluent, and 4–6 per cent. for simple citrate. This blank value observed on adding cell suspension to phosphate buffer must of course be distinguished from the natural hæmolysis due to storage of undiluted citrated blood. The occurrence of such a blank value for hæmolysis on the mere addition of cell suspension to buffer is, of course, evidence of the undesirability of washing stored blood cells before exposing them to lysins.

It will be observed that natural hæmolysis due to storage is most marked with plain citrate solution and least marked with acid glucose citrate, although with all diluents natural hæmolysis increases as the period of storage is prolonged. So too hæmolysis of cell suspensions by bile salts increases with the age of the cells, but hæmolysis by saponin decreases during storage. In view of what has been said before, this paradoxical response will suggest that some substance of the lysolipin-bile salts-soap type had accumulated during storage which decreased resistance to bile salts and increased resistance to saponin, and that this

TABLE XVIII.—EFFECT OF LYSINS ON ERYTHROCYTES OF STORED BLOOD SUSPENDED FOR 1 HR. IN PHOSPHATE BUFFER CONTAINING GLUCOSE 0·5 PER CENT. AND NA FLUORIDE 0·02 PER CENT. AT 23° AND pH 7·3.

Diluent for storage.	Period of storage days.	No. observations.	Natural hæmolysis due to storage, per cent.	Percentage hæmolysis in			
				Bile salts.		Saponin.	
				1/2500.	1/3500.	1/80000.	1/120000.
GC	0	60	0	39±6	21±3·5	93±6·5	70±10
GC	1	17	0	38±5	20±1·5	91±4·5	66±10
PC	14	17	1·6±0·65	65±6	34±6·5	90±6	55±8
GC	14	17	0·5±0·27	59±6	33±4	93±5	62±10
AGC	14	17	0·1±0·04	59±6·5	37±5	89±6·5	53±8·5
PC	28	17	6·5±3	80±4·5	42±9·5	76±11·5	33±8
GC	28	17	2·7±1·5	80±5	42±8	74±13	32±6
AGC	28	17	0·7±0·8	79±6·5	42±9·5	70±11	35±6

PC =Plain citrate =1 ml. sodium citrate +3·5 ml. blood.
GC =Glucose citrate =1 ml. glucose 2·5 per cent. in trisodium citrate 3 per cent. +3·5 ml. blood.
AGC =Acid glucose citrate =1 ml. citric acid, 0·27 per cent.; glucose, 2·5 per cent. in trisodium citrate 3 per cent. +3·5 ml. blood.

same substance may be responsible for the natural lysis observed during storage. This, however, is very unlikely for there is in fact no correlation between susceptibility to bile salts and spontaneous storage-hæmolysis, nor is the latter inversely proportional to lysis by saponin. Thus, after twenty-eight days storage, cells from bloods stored with plain citrate solution showed much more natural hæmolysis than when acid citrate was used, but the susceptibilities of the corresponding cell suspensions to lysins was very similar. The same is true of individual bloods all stored with the same diluent: these may show marked variations in spontaneous hæmolysis, which is not correlated with lysis by hæmolysins (Table XIX).

It is possible that increased susceptibility to bile salts and decreased susceptibility to saponin may be associated with changes in cell volume like those described by Wilbur and Collier [1943]. It is known that cells swell during storage [Maizels and Whittaker, 1939], but alterations in response to lysins would depend primarily not on the size of the stored cells as such but on their size after transference to phosphate buffer containing the lysin. The effect of this on the size of stored cells has not been investigated, but in any case if the changes in response to lysins recorded in Table XVIII are the results of alterations in cell size,

TABLE XIX.—SPONTANEOUS HÆMOLYSIS AND LYSIS BY BILE SALTS AND SAPONIN.

Blood.	Diluent for storage.	Duration of storage.	Natural hæmolysis, per cent.	Percentage lysis after 1 hr. at 23° by			
				Bile salts.		Saponin.	
				1/2500.	1/3500.	1/80000.	1/120000.
1	Plain citrate	28	7·0	84	48	75	28
2	,,　　　　,,	28	6·6	74	41	64	28
3	,,　　　　,,	28	2·9	88	41	78	25
4	,,　　　　,,	28	2·2	78	37	60	24

then the statement that spontaneous hæmolysis is probably not due to accumulation of lysolecithin or some similar substance is confirmed.

Action of Lysins on the Erythrocytes of Anæmic Blood.—The action of lysins on the blood cells of acholuric jaundice has been investigated by Baar and Stransky [1928], but according to Lloyd [1941] the results are conflicting and uninformative. It was for this reason that the present writer has tried to define experimental conditions as rigidly as possible. A major difficulty is the standardization of cell suspensions to be exposed to lysins. Since lysins act at surfaces, the amount of lysin should be adjusted to the surface area of cells exposed. The procedure presents considerable difficulties and is discussed later. In the present investigation suspensions contained a constant dilution by volume of packed cells.

Method.—Blood counts were done on heparinised blood, while fragility tests were done on erythrocytes centrifuged down from a mixture of four parts of blood and one of glucose-citrate solution. Material that had to be transported was carried on ice in a thermos flask along with a normal control. The action of lysins was tested on a suspension which gave 0·01 ml. centrifuged cells in 2 ml. lysin diluted with phosphate buffer at pH 7·3. Lysin was allowed to act for one hour at 25°. Absence of glucose and fluorides and the higher temperature used probably account for the difference between values for normals in Tables XX and XVIII. In Table XX are shown the amount of hæmolysis caused by lysins together with other data.

Cases in Table XX fall into three groups: microcytic anæmias, macrocytic anæmias, and acholuric family jaundice (congenital hæmolytic icterus), each arranged in ascending order of mean cell volume. Normal figures are derived from twenty-seven healthy individuals whose bloods were paired with the anæmic specimens. In the case of lysolipin fragility tests, however, only seven observations were made.

It will be seen that in the case of the microcytic anæmias fragility

TABLE XX.—EFFECT OF LYSINS ON NORMAL AND ANÆMIC ERYTHROCYTES IN PHOSPHATE BUFFERS, 1 HR. AT 25°, pH 7·3.

Case No.	Disease.	Van den Borgh units, indirect.	Hæmoglobin, per cent.	Colour index.	Hæmoglobin, g. per 100 ml. cells.	Mean cell volume, μ^3.	Mean cell diameter, μ.	Per cent. hæmolysis in					
								Bile salts, 1/2000.	Bile salts, 1/2500.	Saponin, 1/150000.	Saponin, 1/180000.	Lysolipin, 1/48000.	Lysolipin, 1/64000.
1	Normals—29 observations Standard deviation	..	..	..	..	..	7·35 ±0·25	42 ±5	29 ±3	69 ±9	54 ±8	66 ±6	24 ±5
2	Carcinoma	..	35	0·52	22	64	7·0	42	21	70	54	..	..
3	Iron deficiency	..	62	0·59	25	66	6·8	54	37	66	56	..	..
4	,, ,,	..	61	0·60	24	70	6·9	46	31	84	74	..	..
5	Hæmorrhage	..	45	0·61	23	74	6·7	49	36	54	40	40	15
6	,, ,,	..	66	0·66	23	76	7·3	50	31	76	65	..	..
7	Gastric ulcer	..	52	0·69	25	77	7·2	52	32	87	67	..	..
8	Rheumatoid arthritis	..	49	0·72	25	80	7·1	39	25	55	44	..	..
9	Vitamine deficiency?	..	25	0·66	21	89	7·6	57	38	74	57	..	..
10	Chronic sepsis	..	55	0·93	27	94	7·7	42	29	86	67	59	25
11	Vitamine deficiency?	0·3	47	0·78	22	96	7·7	68	40	80	66	..	..
12	Toxic jaundice	..	28	0·88	24	102	8·0	69	45	90	83	..	..
13	Aleukæmic leukæmia	0·6	40	1·23	33	103	7·9	53	31	80	58	..	..
14	Non-tropical sprue	3·2	39	1·19	29	112	8·0	64	44	86	70	..	..
15	Pernicious anæmia	1·5	64	1·17	27	126	8·2	80	63	81	70	..	..
16	,, ,,	3·0	26	1·37	27	138	8·5	73	49	83	62	..	..
17	,, ,,	2·5	75	1·52	30	138	8·5	74	50	90	81	87	50
17a	,, ,,	..	78	1·42	29	133	8·3	80	57	92	83	87	42
18	Acholuric jaundice	3·0	66	0·92	32	78	6·1	57	40	77	62	82	46
18a	,, ,,	..	..	..	..	..	..	59	42	78	71	89	54
18b	,, ,,	..	72	0·95	32	80	6·2	61	45	77	66	85	43
19	,, ,,	4·5	85	1·05	34	84	6·2	68	54	78	70	..	..
19b	,, ,,	4·0	76	0·94	34	77	6·2	75	47	82	72	..	..

is normal or slightly increased, but that the findings are not correlated with other data—hæmoglobin, colour index, etc. In the macrocytic group fragility to bile salts and lysolipin is increased, but this is probably not due to the presence of pre-adsorbed lysin of this type since resistance to saponin is also decreased. Erythrocytes in acholuric family jaundice similarly show decreased resistance to bile salts and lysolipin without increased resistance to saponin. It may be wondered if the decreased resistance observed in some of the cases in Table XX were, perhaps, due to decreased protective power of the plasma in disease. In the normal preparations used, intercellular plasma amounted to 0·0002 ml. per 0·01 ml. centrifuged cells, and Table XVII shows that variations in intercellular plasma between 0 and 0·0004 ml. per 0·01 ml. had com-

paratively little effect on lysis. It seems unlikely that the effects of alterations in intercellular plasma in disease will exceed this, but in any case the complication is unavoidable except by repeated washings of pathological cells—a process which in the case of acholuric erythrocytes at least, is undesirable.

DISCUSSION.

It will be realised that the interpretation of data for fragility in cases of anæmia is obscure, for it is difficult to define the standard cell suspension. There are four possible standard suspensions: (*a*) A fixed dilution of blood. This is the method used by Foy and Kondi [1943], following Ponder [1934 *b*] who employed the washed cells from 1 ml. blood in 20 ml. saline. This was satisfactory in the case of the normal bloods used by Ponder, but quite unsuited to work with anæmic bloods where cell suspensions obtained in this way will vary greatly from case to case. Thus in one of Foy and Kondi's experiments, cells in the suspension numbered 134000 per c.mm. compared with 170000 in the control, while in another experiment a suspension contained 215000 acholuric cells compared with a control of 170000 normal cells. Such variations must make the interpretation of results uncertain. (*b*) A suspension containing a constant dilution of packed erythrocytes. This may be compared to estimating the amount of paint needed for decorating a house from the total volume of the house and not from the number of rooms or the surfaces to be covered. It is the method so far used in the present paper and suffers from the defect that surface area per unit volume of cells varies. Thus if 1 c.mm. contain 11 million normal packed cells with a surface area of $134 \mu^2$, total surface area is 1480 sq. mm., while if there are 14·5 million packed anæmic cells per c.mm. with an average area of $113 \mu^2$, total surface area is 1640 sq. mm. per c.mm. cells. (*c*) A suspension containing a constant total surface area. This may be compared to adjusting the amount of paint needed for decorating a house to the total area of the walls. If under these circumstances more or less paint were used than was expected it would be assumed that the quality of the walls was altered so that more or less paint than normal was adsorbed. So too with the constant cell surface suspension: if the cells present were more easily hæmolysed than normal, it would be reasonable to assume that the cells suffered from defective structure or from previous exposure to a lytic agent. (*d*) A suspension containing a constant number of cells. This is analogous to a decorator who estimates the amount of paint needed to decorate a house from the total number of rooms irrespective of their size. It is the method used by Singer [1940] whose suspension always contains 100,000 cells per c.mm. It makes no allowance for surface area which in pernicious anæmia may be 30 per cent. greater than a

suspension containing an equal number of normal cells, so that in pernicious anæmia resistance to lysin might appear to be normal by Singer's method when in fact it was really increased.

It was thought that method (c) was most likely to give useful information, if, as is most probably the case, lysins act at the cell surface and not throughout the whole cell.

The chief difficulty in the way of using suspension (c) lies in estimating the area of the cell surface. This may be done in one of two ways: (i) measuring the amount of a substance adsorbed by constant volume of cells, (ii) by actual measurements. The first method was tried but proved to be unsatisfactory. Of a number of dyes tested, methyl red in equal parts of saline and phosphate buffer at pH 7·3 seemed most likely to satisfy the criteria of adsorbtion in that the amount of dye taken up was far greater than could have existed in simple solution in the cell, combination with the cell was almost immediate—not measurably slow as when a substance penetrates the cell interior—and the amount combined varied inversely with the temperature. On the other hand combination of dye with cells was almost a linear function of the external concentration, though as the latter was increased the amount of dye taken up showed a slight relative increase and not the relative decrease that might be expected in the case of pure adsorbtion. It was expected in the microcytic anæmias which have a relatively large area in unit volume of cells, that methyl red taken up would be greater than normal, but actually it was less, indicating that the dye was not adsorbed evenly over the surface but only at specific points whose total area is decreased in spite of the increase of total surface area in unit volume (Table XXI).

TABLE XXI.—DYE TAKEN UP BY ERYTHROCYTES IN 50 VOLUMES OF METHYL RED, 100 MG. PER CENT. IN SALINE-PHOSPHATE BUFFER FOR 5 MINUTES AT 25°, pH 7·3.

Cases.	Number.	Mean cell volume average, μ^3.	Dye taken up by cells, mg./100 ml.
Normals . . .	29	85±4	347±13
Microcytic anæmia .	8	74	299
Macrocytic anæmia .	10	113	307
Acholuric jaundice .	3	78	332

In the case of the macrocytic anæmias the expected decrease of methyl red adsorbtion is found, but there is now correlation between cell volume and dye adsorbed.

With regard to estimating surface area from cell measurements, two methods are available, both approximate: (a) Haden [1931] has shown that except in the case of acholuric jaundice, the erythrocyte retains

its normal proportions whatever its size.   In this case, if

a = total area per c.mm. patho-   and A = total area per c.mm. normal
     logical cells   cells,

n = number pathological cells   and N = number normal cells per
     per c.mm.   c.mm.,

v = mean cell volume of patho-   and V = mean cell volume of normal
     logical cells   cells,

then
$$a/A = n/N \times \sqrt[3]{(v/V)^2},$$

and since the number of cells per c.mm. = 1/mean cell volume,

$$a/A = V/v \times \sqrt[3]{(v/V)^2} = \sqrt[3]{V/v} = Index\ I.$$

(b) The second method requires one to assume with Emmons [1927-28] that the erythrocyte is a flat cylinder.   In this case,

the area of a single cell = 2(π radius^2 + cell volume/radius).

If r = average radius of pathological cells and R = average radius of normal cells then

$$a/A = n/N \times \frac{\pi r^2 + v/r}{\pi R^2 + V/R} = V/v \times \frac{\pi r_2 + v/r}{\pi R^2 + V/R} = Index\ II.$$

Index II requires a measure of mean cell diameter and this has been obtained by an approximate halometric method [Price, 1929] with a personal error of $\pm 0.1\mu$ when five observations are made on a single blood film.   The halometric method is not directly referable to Index II since it employs a dry film, while the measurement of mean cell volume is done on fluid blood.   However, the work of Haden [1935] suggests that the diameter of the dried cell is closely related to that of the wet cell and certainly Indexes I and II agree well in spite of the techniques employed—except in the case of acholuric jaundice where the cells are disproportionately plump and where Index I cannot properly be applied (Table XXII, columns 3 and 4).   In adjusting the dose of lysin to surface area, therefore, it was thought desirable to use Index II which is more generally applicable.

It is now possible to prepare three standard suspensions: (B) the volume of normal or anæmic cells in a suspension is kept constant — 0·1 ml. in 5 ml.   (C) The total surface area in the suspension is kept constant.   (D) The number of cells in unit volume is kept constant.   To obtain C and D, it is necessary in the case of each blood to put up several different dilutions of cells, draw curves of the amounts of hæmolysis observed with each dilution of lysin and then mark off the amount of hæmolysis corresponding with the suspension in question.   Thus in case 17, the suspension by volume is 0·1 ml. in 5 ml. phosphate buffer. But the surface area in 0·1 ml. centrifuged cells is only 85 per cent. that of normal centrifuged cells, hence the suspension employed for C should contain 0·1 ml. cells in 5 × 0·85 = 4·25 ml.   Again, to secure a suspension

containing a constant number of cells it will be necessary to suspend 0·1 ml. centrifuged cells in $5 \times 85/138$ ml., since normal cell volume is $85 \mu^3$, while that of the anæmic cell in this case is $138 \mu^3$. One therefore marks off on the curves the amounts of hæmolysis produced by standard dilutions of lysins on 0·1 ml. cells in 5, 4·25, and 3·1 ml. buffer and this gives the data B, C, and D respectively in Table XXII.

It will be seen from Table XXII that lysis by saponin is not greatly affected by the strength of the cell suspension, and this is what might be

TABLE XXII.—FRAGILITY TO LYSINS OF SUSPENSIONS CONTAINING (B) CONSTANT VOLUME OF CELLS PER UNIT VOLUME OF SUSPENSION. (C) CONSTANT SURFACE AREA PER UNIT VOLUME. (D) CONSTANT NUMBER OF CELLS PER UNIT VOLUME.

Case.	Mean cell volume.	Ratio cell areas per ml., anæmia/normal.		Per cent. hæmolysis by								
		Index I.	Index II.	Bile salts, 1/2000.			Saponin, 1/180000.			Lysolipin, 1/48000.		
				B	C	D	B	C	D	B	C	D
Normal	85	1	1	42 ±5	42 ±5	42 ±5	54 ±8	54 ±8	54 ±8	66 ±6	66 ±6	66 ±6
2	64	1·10	1·14	42	45	58	54	56	57	..	..	..
3	66	1·09	1·10	54	61	76	56	53	48	..	..	..
4	70	1·07	1·06	46	48	54	74	74	73	..	..	..
5	74·	1·05	1·02	..	..	..	..	..	..	..	..	..
6	76	1·04	1·06	50	53	56	65	66	68	..	..	..
7	77	1·03	1·04	52	53	55	67	66	66	..	..	..
8	80	1·02	1·01	..	..	..	..	..	..	..	..	..
9	89	0·98	1·00	57	57	57	57	57	57	..	..	..
10	94	0·95	0·98	42	40	36	67	66	66	59	59	50
11	96	0·96	0·96	68	64	52	66	66	66	59	58	52
12	102	0·94	0·96	69	67	59	83	81	75	..	..	..
13	103	0·94	0·95	..	..	..	..	..	..	..	..	..
14	112	0·91	0·91	64	58	44	70	69	67	..	..	..
15	126	0·87	0·86	80	74	62	70	72	75	..	..	..
16	138	0·85	0·85	73	60	36	62	64	66	..	..	..
17	138	0·85	0·85	74	58	32	81	78	73	87	74	59
17a	133	0·86	0·85	80	68	42	83	79	74	87	72	53
18	78	1·03	0·91	57	51	64	62	60	63	82	74	88
18a	80	1·02	0·91	61	52	68	66	63	67	85	78	88
19	84	1·00	0·89	68	62	68	66	63	67	..	..	..
19b	77	1·03	0·92	75	70	80	47	42	52	..	..	..

expected from Table II of Part I. On the other hand, there is a general increase of susceptibility to saponin, more especially in the macrocytic group; in the microcytic cell group figures are more variable but are also slightly greater than normal, and the same is true of acholuric cells. In the case of bile salts and lysolipin, method B suggests that resistance is normal or slightly increased in the microcytic group and this suggestion is slightly emphasised when a correction for surface area is made (C).

The use of a suspension containing a constant number of cells (D), on the other hand, indicates a definite increase in fragility. In the case of the macrocytic anæmias, method B suggests definitely increased lysis by bile salts, and lysolipin and lysis is also apparently increased in method C. According to method D, however, resistance to bile salts and lysolipin would appear to be normal or only slightly increased. This agrees with Singer [1930] who used method D and records normal fragility to lysolecithin in pernicious anæmia, although in fact his normal cells averaged 27 per cent. hæmolysis and his macrocytic cells 36 per cent. In the acholuric family jaundice cases, fragility is definitely increased to bile salts and lysolipin by method B, less so by method C, where correction has been made for lack of surface area in unit volume of cells. On the other hand, by method D and in agreement with the findings of Singer and of Lloyd, fragility appears to be very definitely increased to bile salts and lysolipin. It must be emphasised, however, that in suspensions prepared by the methods of Singer and Lloyd total surface area varies widely, the ratios in cases 1, 2, 17, and 19 a (Table XXII) being 1, 0·72, 1·62, and 0·91 respectively, and much of the variation in the observed hæmolysis may be due to quantitative differences in the extent of cell surface, rather than to qualitative alterations. For this reason, it is thought that suspensions containing constant total surface area alone give comparable results.

With regard to the above data, comparable results are obtained with other dilutions of each lysin. But even though one accepts the principle of using suspension containing constant total surface area, there remains still another difficulty in the way of interpretation: as lysis proceeds, it tends to be arrested by combination of the lysin with lipins in the cell envelope and also by hæmoglobin shed from lysed cells. The latter factor is much less important but is not insignificant. Since microcytic cells liberate less hæmoglobin than normal cells, lysis would, apart from all other factors, tend to proceed further than with normal cells. Bearing this in mind, it is probably correct to say of the microcytic anæmias, that resistance to bile salts is probably increased in cases 2 and 6, decreased in case 3, and normal in the rest. In the macrocytic anæmias resistance to bile salts is probably normal in case 10 and also in case 9 where inhibition by hæmoglobin liberated will be only two-thirds of normal. It is almost certainly decreased in cases 13, 14, 15, 16, and 17 where inhibition of lysis by hæmoglobin is about normal. The effects of lysolipin are similar to those of bile salts. It is improbable that this increased susceptibility to lysis by bile salts and lysolipin is due to a pre-adsorbtion of a lysolipin-like substance in vivo, for had such an adsorbtion occurred resistance to saponin would have been increased, which was not the case.

In the case of acholuric cells, it is probable that resistance to bile salts and lysolipin is somewhat decreased, though not to the extent that

the data of Singer, uncorrected for surface area, suggest. As in the case of the macrocytic anæmias, there is no suggestion that lysin pre-adsorbed *in vivo* plays any part in the increased fragility to bile salts and lysolipin, and in both conditions it is likely that this increased fragility is due to some deficiency of protective substances present in greater amounts in the cells of normals and microcytic anæmias.

CONCLUSIONS.

In stored blood, susceptibility to lysis by bile salts and lysolipin is increased, while to saponin it is decreased. This suggests that some substance with certain of the properties of lysolipin, bile salts, and soaps has appeared during storage, but it is thought unlikely that this substance is responsible for the natural hæmolysis occurring during storage, for the altered response is not correlated with natural hæmolysis.

In the microcytic anæmias, lysis by bile salts, lysolipin, and saponin is probably normal. In acholuric family jaundice resistance to these lipins is somewhat decreased, while in the microcytic anæmias it is definitely decreased. There is no evidence that this decrease of resistance is due to the pre-adsorbtion of a hæmolytic substance *in vivo*.

If experiments on hæmolysis are to have any meaning, the titres of lysins used ought to be referred to the total surface area of the cell suspensions.

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A BIOCHEMICAL STUDY OF ISOLATED PERFUSED LUNGS WITH SPECIAL REFERENCE TO THE· EFFECTS OF PHOSGENE. By I. DE BURGH DALY, P. EGGLETON, S. R. ELSDEN, and CATHERINE O. HEBB. From the Physiology Department, University of Edinburgh.

· THE aim of the investigation to be described has been:—

(1) To discover the direct effects of phosgene on lungs uninfluenced (*a*) by the complex cardio-respiratory responses which phosgene produces in the entire animal, and (*b*) by potential toxic substances which might be formed elsewhere in the body and be carried by the blood to the lungs.

(2) To determine how far the blood changes in phosgene-gassed animals described by other workers are due to the direct action of phosgene on the lungs.

Such an investigation appeared to us to be a necessary step in the elucidation of the precise effect of phosgene on the lungs since, when acting in the entire animal, phosgene causes early reflex cardiovascular and respiratory responses, responses which give rise to pulmonary events masking the initial direct action of phosgene on the lungs.

In the experiments to be described the conditions necessary for the maintenance of constant pulmonary blood flow and of constant forces applied to the lungs for the purpose of ventilation limited the duration of the experiment to not more than seven hours. The results to be described therefore refer only to pulmonary events during the seven hours or less subsequent to gassing.

METHODS.

Perfusion Technique.

The lungs of dogs are isolated from the body and perfused under negative pressure ventilation with heparinised blood, the blood inflow being kept constant. In some experiments the lungs have been separated, one lung being used as a control, the other as the test object. These will be referred to as separated isolated perfused lungs. The methods are fully described by Daly, Hebb, and Petrovskaia [1941]. Continuous measurements are made of the pulmonary arterial pressure and tidal

air. The blood flow is kept constant at a flow equivalent to 50 c.c./min./kg. body-weight of the animal from which the lungs are taken. The extra-pulmonary pressure variations for ventilation are *ca.* $+0.5$ to -12 cm. H_2O.

Phosgene Administration and Estimation.

A diagram of the closed respiratory system is shown in fig. 1. The tracheal cannula is fitted with inspiratory and expiratory valves which ensure a flow of air through a cold spiral (A), the volume of which is
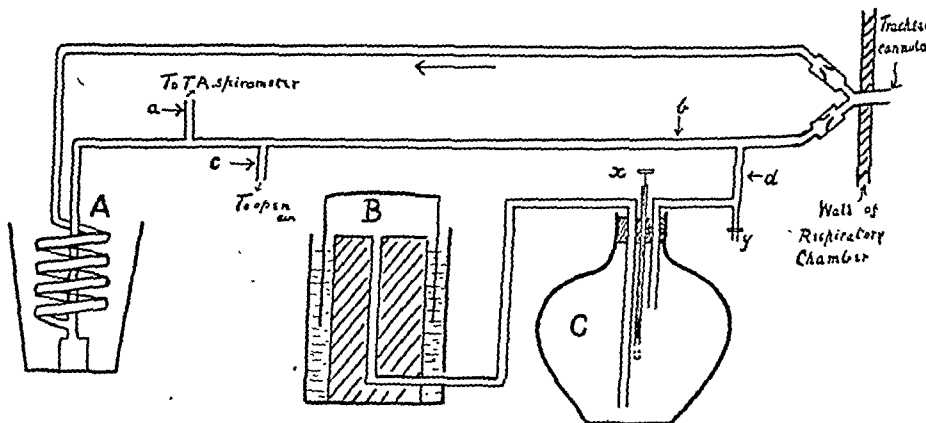

Fig. 1.—Arrangements for gassing with phosgene and for collection of expired air mixture.

A = pipe coil with water trap in ice bucket.
B = spirometer.
C = carboy fitted with brass cylinder and piston (x) for breaking phosgene capsule.
y = sampling tube connexion.
During non-gassing period channels at "a" and "b" are open, at "c" and "d" closed.
During gassing periods channels at "a" and "b" are closed, at "c" and "d" open.

greater than the tidal volume of the lungs, for the purpose of condensing expired water vapour. The tidal air is taken by means of a small spirometer. Before and after the gassing period, the system at "a" and "b" is open, at "c" and "d" closed. During gassing, "a" and "b" are closed, "c" and "d" open, so that the phosgene in the carboy (C) is inspired into the lungs, the carboy mixture being diluted with air from the large spirometer (B) during the procedure.

The carboy is charged with phosgene by placing the glass capsules containing the liquid phosgene (30 mg. or 150 mg.) in a long brass cylinder which passes through the carboy bung. The cylinder is fitted with a piston (x) an extension of which projects outside the carboy. The glass capsules are smashed against the end of the cylinder by giving the piston extension a sharp tap. The gas escapes through holes in the end of the cylinder.

The estimation of phosgene was based on the reaction between excess aniline-water and phosgene producing the slightly soluble diphenyl urea.

$$COCl_2 + 4C_6H_5NH_2 \rightarrow {C_6H_5NH \atop C_6H_5NH} \hspace{-0.3em}\Big\rangle CO + 2C_6H_5NH_2, HCl.$$
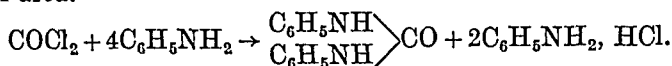
The amount of diphenylurea, and thus the amount of phosgene, was obtained by determining the total N, by the micro Kjeldahl technique, of the precipitate, 1 mol. of phosgene being equivalent to 2 mols. ammonia [Eggleton and Elsden, 1941].

The procedure was as follows: a sample of the air containing phosgene was withdrawn from the gas reservoir by means of an exhausted Buchner flask of known volume. The flask was then connected to a precipitation tube containing 10 c.c. of aniline water saturated with diphenyl urea and its contents displaced through the aniline water by a slow current of air. When precipitation was complete the tube was disconnected and the diphenylurea collected by suction on to a small pad of acid washed asbestos pulp supported on a small glass bead in a 1-inch funnel. This form of micro filter is to be recommended (see Peters and van Slyke, 1932). The precipitation tube and precipitate were then very thoroughly washed with N/1 HCl saturated with diphenylurea to remove contaminating aniline, and the precipitate transferred to the combustion tube by inverting the funnel and dislodging the glass bead and asbestos pad with a thin glass rod. Any diphenylurea adhering to the precipitation tube or the funnel was taken up in hot alcohol and added to the remainder of the precipitate. The alcohol was evaporated off on a water-bath and the total N of the residue determined as described above.

Blood Examination.

Throughout the experiment blood samples have been taken before and after the administration of phosgene and the following measurements made.

(a) *Red and White Cell Count.*—The cell counts were carried out in the usual manner with a Neubauer hæmacytometer. In our early experiments we found that when the blood concentration is high, the red cell counts appear to be unreliable, probably owing to the difficulty of mixing the blood in the presence of red cell aggregations. In later experiments we therefore relied upon the hæmoglobin-iron and hæmatocrit measurements only as an indication of blood concentration. Cell counts will not be referred to again.

(b) *Diameter of Red Blood Cells.*—Dry blood films were examined with a Zeiss $\frac{1}{12}$-inch objective and a scale ocular No. 6. One hundred cells were counted in each film, the crenated and misshapen cells, which were generally smaller than normal, being neglected. A distri-

bution curve of cell diameters was plotted and the median computed. According to Ponder [1934] the diameter of the cell in dry films is 8–16 per cent. less than that of cells floating in plasma. Since we were interested in relative rather than absolute changes, this was not a serious drawback for our purpose.

(c) *Blood Sedimentation Rate.*—This was measured at room temperature in a graduated tube of 1·8 mm. diameter. The results were computed in the usual way, *i.e.* the B.S.R. was taken as the mean hourly velocity of sedimentation for the first, second, and third hours.

(d) *Hæmatocrit.*—The hæmatocrit determinations were made by the method described by Meyerstein [1942] with tubes of 0·2 c.c. capacity centrifuged at 3500 r.p.m. for 15 minutes or with tubes of 0·1 c.c. centrifuged at 10,000 r.p.m. for 10 minutes. This method was found to be reliable, duplicate determinations even with the smaller tubes differing by less than $\pm 0·5$ per cent., the error generally being in the region of $\pm 0·2$ per cent. All determinations were made in duplicate and the mean recorded.

(e) *Hæmoglobin Iron (H.F.).*—This was estimated in 0·1 c.c. of blood by converting it to cyan-hæmoglobin according to the method of Stadie [1920] and Wu [1922], as described by Peters and van Slyke [1932]. The intensity of the colour was determined with the aid of a Hilger Spekker photoelectric absorptiometer, using a green filter (No. 5), and the amount of hæmoglobin iron obtained by reading off from a standard curve which had been derived from a series of bloods of predetermined oxygen capacity on the basis that 55·8 mg. hæmoglobin iron (1 mg. atom) are equivalent to 22·4 c.c. oxygen (1 mg. mol.). To convert hæmoglobin iron into hæmoglobin it is assumed that the molecular weight of hæmoglobin is 67,000 and that 1 molecule of hæmoglobin contains 4 atoms of iron. Thus 1 mg. hæmoglobin iron is equivalent to $\dfrac{67,000}{4 \times 55·8} = 300$ mg. hæmoglobin. For the construction of this curve human blood and the blood of rabbits and dogs were used, and substantial agreement obtained for the three species.

(f) *Viscosity of Blood and Plasma.*—Most of the measurements were made at or near 18° C. with an Ostwald viscosimeter of 4 c.c. capacity, in which water took 27 sec. to fall and whole blood about 120 sec. Earlier measurements were made with a rotary cup containing a suspended bob (Couette method). The cup was rotated continuously by a synchronous A.C. motor and the angle of twist of the bob recorded optically. This method gave results in agreement with the Ostwald and was later abandoned in favour of the latter. Ostwald results were corrected for density differences. Since the blood viscosity was measured at room temperature, the values obtained were higher than those actually present in the circulating blood at 37° C. Even so the ratio of the initial to the final viscosity values at room temperature

will not differ from the corresponding ratios at 37° C. provided there are no serious alterations in plasma tonicity. These conclusions are largely based on a large number of control measurements since made by one of us (C. O. H.) in work which it is hoped will shortly be published. The paper by Rothlin [1920] on the effect of temperature on blood viscosity might also be consulted. Except in experiment 33 the tonicities show little change (Table I) and thus the percentage differences in viscosity found for room temperature may be taken as giving a good approximation of the corresponding changes at the circulating blood temperature.

(g) *Specific Gravity of Blood and Plasma Measured by Falling Drop Method.*—The speed of fall of a drop of liquid of density ρ_1 and radius r through an immiscible fluid of density ρ_2 and viscosity η is given by Stokes's Law as

$$\frac{2g(\rho_1 - \rho_2)r^2}{9\eta},$$
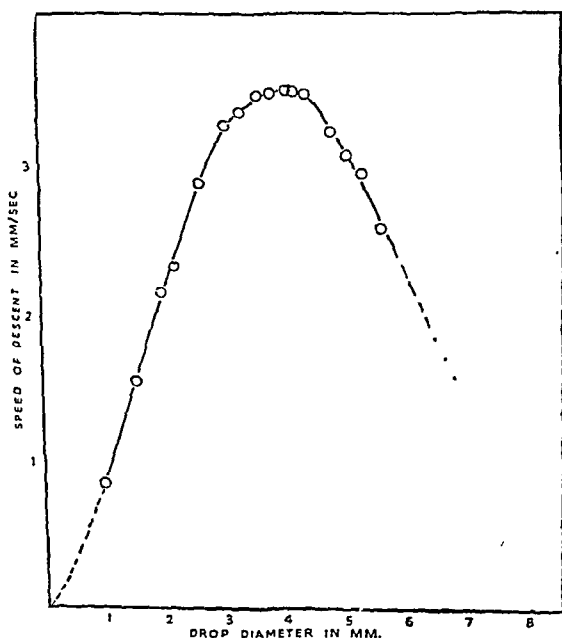
Fig. 2.—Speed of fall of drops of 2·32 molar NaCl in paraffin-pentachlorethane mixture at 25° C. Burette inclined 1° to vertical. Diameter of burette 8·6 mm.

provided the containing vessel is very wide compared with the diameter of the drop. If a vertical cylindrical tube is used it is found that the rate of fall is maximal for a drop of diameter near to half that of the containing cylinder (fig. 2). The ratio is not critical, and for drops

TABLE I.—ISOLATED PERFUSED LUNGS. VALUES OF VARIOUS FACTORS AS PERCENTAGE OF INITIAL VALUES.

Experiment	Time of initial and final samples		T.A.	P.A.p.	r.b.c. haematocrit	Hæm. Fe.	w.b.c. cu. mm.	Viscosity		Sp. gravity		Con-ductivity		NaCl.		Urea N.	N.P.N.	Total N.	Plasma protein	Cl protein
								Blood	Plasma	Blood	Plasma	Blood	Plasma	Blood	Plasma					
27	0′, 280′	P	7	97	..	121	..	167	90	..	..	85	110	99	99	108	213	110	106	91
28	0′, 267′	P	33	105	..	116	7	63	63	..	..	98	106	100	99	108	106	109	112	95
29	0′, 220′	P++	4	97	..	123	8	114	88	..	..	78	95	101	104	108	104	114	112	93
30	45′, 259′	P+	14	104	155	158	..	163	101	100·8	100·2	33	..	87	103	100	150	113	112	92
32	50′, 350′	C	1	130	127	122	46	241	114	101·0	100·2	78	101	93	98	99	..	114	118	84
R 33	67′, 250′	C	9	228	126	148	..	490	190	101·0	101·2	36	109	92	116	124	..	152	..	76
L 33	67′, 250′	C	9	165	123	130	..	226	138	101·0	100·9	64	112	100	118	122	..	130	..	90
R 34	-52′, 316′	C	8	208	168	108	..	720	..	102·7	100·7	21	97	83	108	108	..	..	..	
L 34	-52′, 316′	C	8	258	160	100	..	492	..	102·3	100·7	35	101	88	114	130	107	..	..	
R 35	17′, 405′	P+++	12	136	105	120	..	179	178	100·6	101·0	78	92	104	107	104	107	156	158	68
L 35	17′, 405′	C	31	146	103	116	..	212	216	100·6	101·3	82	84	102	107	102	104	164	167	64

R=right, L=left lung; T.A.=tidal air; P.A.p.=pulmonary arterial pressure; N.P.N.=non-protein nitrogen; P=phosgene; C=control. The time of initial and final samples is given in relation to the start of perfusion at 0. Thus in experiment 34 the initial blood samples were taken 52 minutes before perfusion started, the final after 316 minutes of perfusion.

within 10 per cent. of this relative size the speed of fall is practically independent of exact size.

As containing vessel we used a 10-c.c. burette of diameter 8·6 mm. for which the correct drop size was 35 mm.[3] (4 mm. diam.). To obtain speeds of fall of a convenient order, medicinal paraffin was used, to which was added sufficient pentachlorethane to give a density of about 1·02 (carbon tetrachloride is too volatile for this medium, which is open to the air when in use). The time taken to fall between the zero and 10 c.c. marks on the burette (170 mm.) was of the order of 500 sec. for plasma and 100 sec. for whole blood. The apparatus (which was kept at $25° \pm 0·1°$ C. in a 1-litre measuring cylinder) was calibrated by means of NaCl solutions of known density. This arrangement has one drawback where great accuracy is needed: the falling drop may "wander" near to the wall of the cylinder, when its speed becomes somewhat greater. We therefore kept the burette permanently inclined at an angle of about 1° so that the drop always followed the same path.

The absolute accuracy of the method depends on the accuracy of the NaCl solutions used for standardising and of the density tables used. In our case it is probably not more than 1 in 1000. But the accuracy of a group of results, relative to any one of them, is much greater, for the quantity measured is the small excess of density of the test drop over the disperse medium, and this excess is easily measured to one part in 500. It is therefore possible to distinguish two aqueous solutions differing in density by as little as 0·00002.

(h) *Chlorides of Blood and Plasma.*—Electrometric method. (For details, see Eggleton, Eggleton, and Hamilton, 1937.) A preliminary removal of protein was found necessary because some constituent of red cells, removable by trichloracetic acid, interferes with the silver electrodes. The results are expressed as mg. Cl/c.c.

(i) *Conductivity of Blood and Plasma.*—Measurements were made on 4 volts, 50 cycle A.C., between silver electrodes 50 mm. apart in a horizontal tube of 2 mm. bore, using a standard Cossor ohmmeter. No capacity or inductance large enough to affect 50 cycle current could be observed in the system. The measured resistance of plasma was of the order of 10,000 ohms, and of blood 35,000 ohms. The current density was therefore less than 12 m.a. per cm.[2] The instrument was calibrated on NaCl solutions and all results expressed in terms of the NaCl solution having the same resistance at the same temperature.

(j) *Urea.*—By the method of Conway [1939].

(k) *Total Nitrogen and Non-Protein Nitrogen.*—Total nitrogen was estimated on 0·2 c.c. samples of plasma and the N.P.N. on 2 c.c. samples. Trichloracetic acid was used for removal of protein.

The combustions were achieved in 6-inch Monax glass boiling tubes using 1 c.c. of digestion mixture made up as follows:—

3 parts conc. H_2SO_4,
1 part syrupy H_3PO_4,
0·5 part 5 per cent. solution $CuSO_4.5H_2O$.

The digests were finally cleared by adding to the cooled mixture 1 c.c. distilled water and about 0·5 g. A.R. potassium persulphate and bringing to the boil. The digests were then transferred to the distilling apparatus, made alkaline with 40 per cent. soda and the ammonia trapped in 10 c.c. of 2 per cent. boric acid containing Tashiri's indicator. A litre of the boric acid contained 10 c.c. of stock indicator which was made up according to Cole [1933].

After distillation the boric acid solution containing the ammonia was brought to the boil and titrated hot with *ca.* 0·02N HCl back to the original colour, the amount of HCl used being equivalent to the amount of ammonia present. This method [Woolf, 1927] gives a greater range than the back titration method with no corresponding loss of sensitivity.

(1) *Protein.*—The protein content of plasma was computed from estimations, by the micro Kjeldahl technique, of the total N before and after removal of protein by 10 per cent. trichloracetic acid on the assumption that protein $N \times 6·25 =$ protein.

RESULTS.

The Action of Phosgene on Isolated Perfused Lungs.

Since it was our aim to compare the course of events in one lung ventilated with air with that taking place in the other lung gassed with phosgene, it became necessary to discover how far one lung differed from the other during perfusion, neither being gassed with phosgene. Experiments 33 and 34 of figs. 3A, 3B and Table I show the order of the differences in the various factors measured. They require some comment. The lungs in experiment 33 expanded poorly at the outset and rapidly lost their elasticity, as indicated by the steep fall in tidal air in a comparatively short time. At the time we attributed this to a dry visceral pleura and to the unusually early onset of hæmolysis during perfusion. It is to be noted that the progressive decline in tidal air of each lung, although separately perfused, follows approximately a similar time course. In contrast to experiment 33, the lungs of experiment 34 appeared to be in moderately good condition at the start of perfusion, and here again the progressive changes in tidal air of each lung do not show marked differences.

Without at the moment entering upon a discussion of the significance of the direction of change during perfusion of the other factors measured, we wish to stress the order of the differences in the physico-chemical properties of the blood found between each lung of experiments 33 and 34. Figs. 3A, 3B show these quite clearly. They can be used for

comparison with experiment 35 in which one lung was perfused and ventilated with air (broken line) and the other lung perfused and gassed with phosgene (continuous line). It will be seen that the only significant difference between the control and gassed lung of experiment 35 is the more rapid reduction in tidal air of the gassed lung. All the other factors of this experiment illustrated in figs. 3A, 3B show no greater differences between the control and gassed lung than are found between each lung of experiments 33 and 34.

What is not shown in figs. 3A, 3B is that two other differences between the control and gassed lung were discovered, namely, that phosgene produced desquamation of the bronchial epithelium which was absent in the control lung (fig. 4, *a*, *b*) and an increased circulating blood volume of the lungs. The histological changes in non-gassed isolated perfused lungs have already been described by Trowell [1943]. The evidence for an increased circulating blood volume of the lungs was obtained in the following manner. In experiment 35 it was found that the volume of blood expressed from the gassed lung when inflated at the end of perfusion was equal to 135 per cent. of the weight of lung tissue, while the corresponding value for the control lung was 74 per cent. This order of difference has been consistently found in subsequent experiments in which the measurement has been made. The blood obtained in this way at the end of perfusion is regarded as being derived chiefly from capillaries which were active during perfusion because the lungs became pale on inflation. Inflation of the dark red œdematous lung in which there are blood infarcts does not become pale on inflation. The problem will be referred to in a later paper.

We may conclude then that in perfused lungs isolated from the nervous system and systemic circulation (bronchial vascular system) phosgene, in sufficient concentration to produce a marked reduction in tidal air, bronchial epithelium desquamation, and an increase in the circulating blood volume of the lungs, causes no significant changes in the pulmonary arterial pressure or in the physico-chemical properties of the blood which we measured as compared with those taking place in control perfused lungs. This somewhat unexpected result requires further comment in relation to the phosgene concentrations used and its site of action on the lungs.

In experiments 31 and 35 the phosgene concentration in the reservoir was measured at the beginning and end of the gassing period (Table II). In the remaining experiments the initial concentration was computed from the amount stated by the manufacturers to be in the glass capsule. The rate of hydrolysis and of adsorption on the glass and rubber surfaces of the apparatus was not known, nor was the rate of reduction in tidal air during the gassing period known since the tidal air was not taken during this period. It is not possible therefore to give values of phosgene concentrations entering the trachea. An approximate
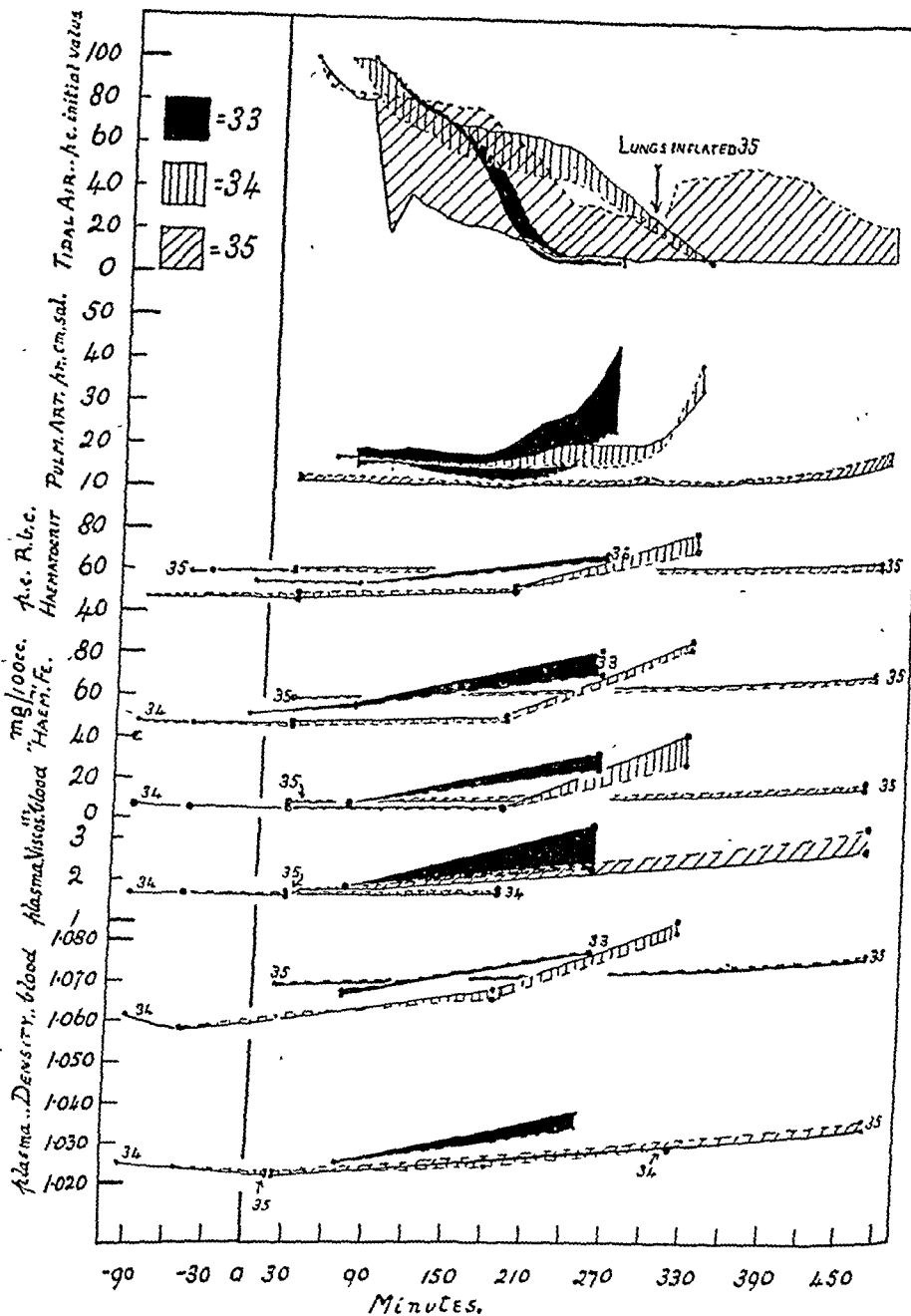
FIGS. 3A, 3B.—Separated isolated perfused lung preparations showing differences when each lung is used as a control (experiments 33, 34), and when one lung is used as control and the other gassed with phosgene (experiment 35).

The continuous line in each experiment represents the values obtained for one lung and the broken line those obtained for the other. The solid and hatched
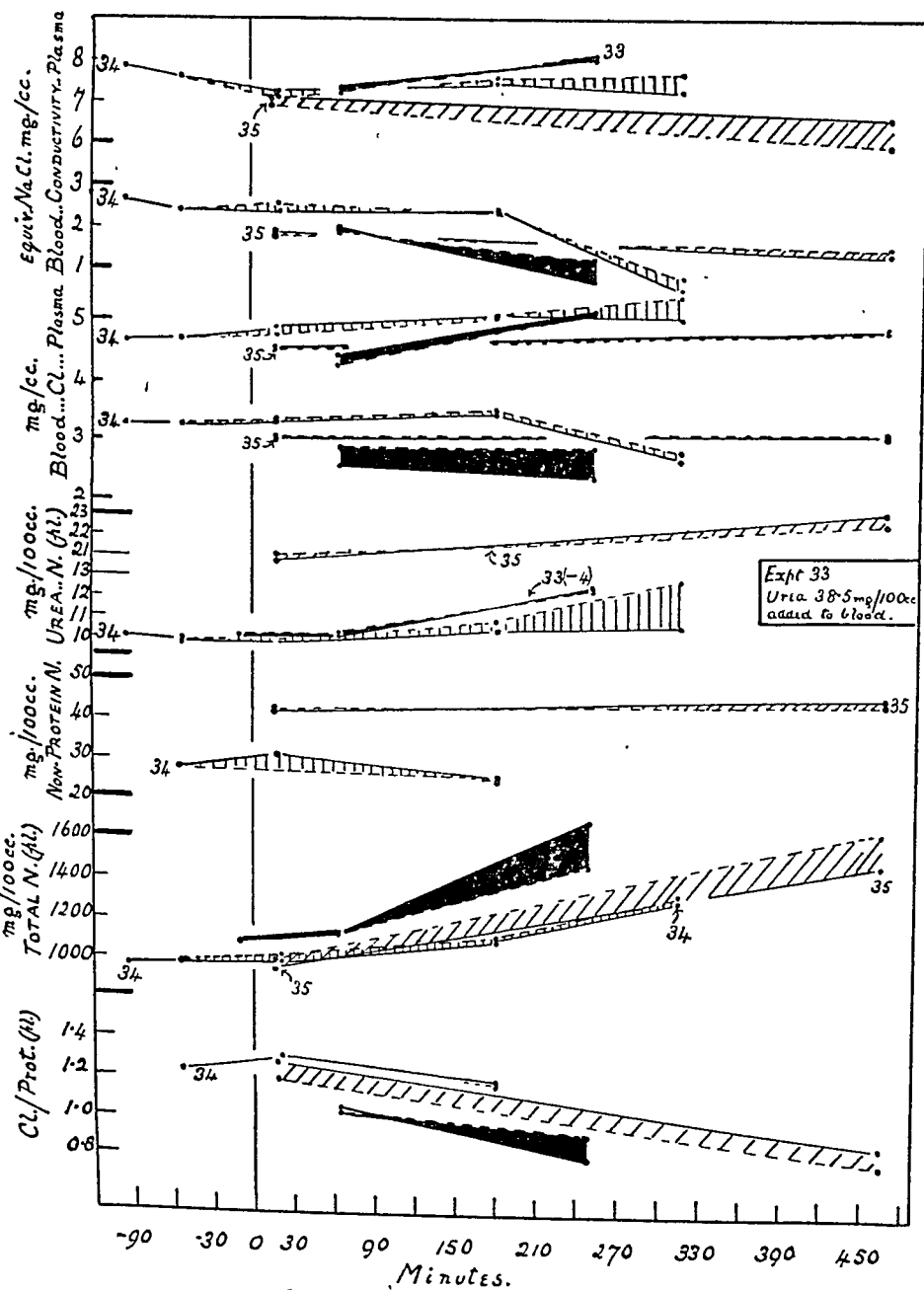
FIG. 3B.

areas between these lines facilitate the appreciation of the differences obtained between each lung. In experiment 35, continuous line = gassed; broken line = control lung.

Zero time corresponds to the commencement of perfusion. Preperfusion period samples of blood were taken from the glass vessel used for collecting the blood from the femoral artery.

Table II.—Phosgene Concentrations and Tidal Air (T.A.) Reduction during Administration of Gas.

Expt.	Phosgene conc. mg./cu.m.		t. min.	$\dfrac{Ct}{1000}$	T.A. at end of gassing period as per cent. initial value.
	Initial (C_1).	Final (C_2).			
27a	610 *	n.t.	1·7	(1)	100
b	610 *	n.t.	4·5	(2·5)	96
28	610 *	n.t.	6·0	(3)	96
29	3,050 *	n.t.	23·5	(41)	25
30	3,050 *	n.t.	8·7	(21)	63
31	4,000	980	27·0	57	35
35	11,500	3,800	20·0	140	47

n.t. =not taken; experiments 31 and 35 suggest an exponential loss of phosgene by hydrolysis and adsorption on glass and rubber surfaces with a time constant of 18·5 minutes.

* =amount estimated by manufacturers to be in capsule.

Approximate values for Ct in experiments 27, 28, 29, and 30 were obtained on the assumption that the phosgene loss progresses exponentially at rate found in experiments 31 and 35. On this basis the value of Ct is obtained from the expression $Ct = 18\cdot5\ C_1(1 - e^{\frac{-t}{18\cdot5}})$. The figures are only an approximate guide.

comparative estimate can probably be reached on the assumption that hydrolysis proceeds uniformly (Table II). Consideration of this Table suggests that in experiments 29, 30, 31, and 35 a heavy concentration of phosgene existed in the trachea; indeed we know it was sufficient to cause desquamation of the bronchial epithelium. The absence of physico-chemical changes in the blood may have been due to bronchiolar constriction preventing high concentrations of phosgene from reaching the alveoli and alveolar capillary surfaces. That some phosgene did reach the alveoli is suggested by the increase in circulating blood volume of the gassed lungs. It was, however, not sufficient to produce gross capillary damage, for after the blood had been expressed from the lungs by inflation histological examination revealed no significant differences between the alveolar cells and capillaries of the control and of the gassed lungs.

We would point out that bronchiole constriction in isolated perfused lung preparations under negative pressure ventilation is more effective in reducing the "phosgene acceptance" of the lungs than it is in the living animal. In the lung preparations the forces expanding the lungs remain constant during the gassing period, whereas in the living animal the increased respiratory effort tends to compensate for any bronchoconstriction which may ensue.

These considerations lead us to suggest that under the conditions of our experiments the bronchiolar spasm during gassing confines the major pathological changes to the bronchial tubes and affords some
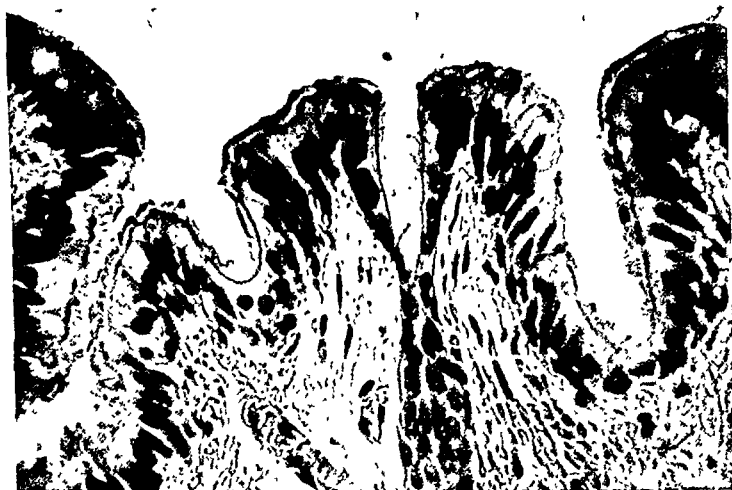
(*a*) Control lung. × 465.   Bronchial epithelium normal.
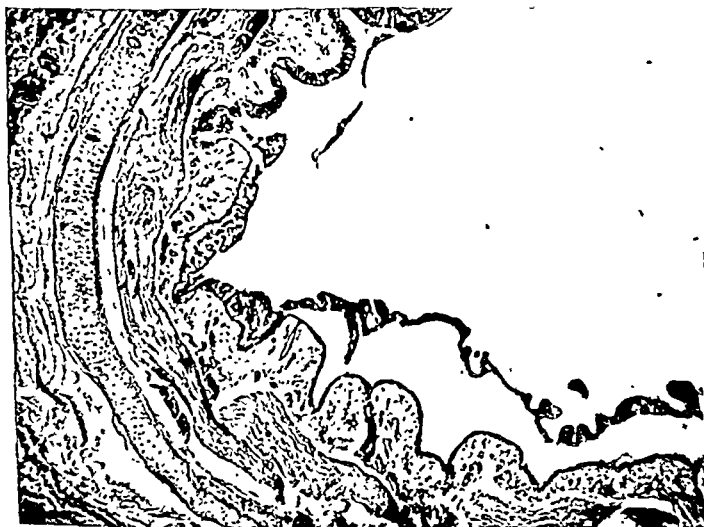
(*b*) Phosgene-gassed lung. × 55.   Desquamation of bronchial epithelium.

FIG. 4.—Experiment 35.

· protection to the alveoli. Alternative interpretations of our results will be considered after changes in the blood of control perfused lungs have been described more fully.

Blood Changes in Non-Gassed Isolated Perfused Lungs.

Before examining these in detail it should be mentioned that the experimental procedure was not precisely similar in all the experiments. In the particular form of negative pressure ventilation used there is a continuous passage of air through the respiratory chamber which tends to cause dryness and stiffness of the visceral pleura with a consequent reduction in tidal air. The admission of steam to the chamber keeps the pleura moist and overcomes this disadvantage [Daly, Hebb, and Petrovskaia, 1941]. In experiments 27–33 no steam was used, in experiments 34 and 35 it was admitted to the chamber. In experiment 29 the lungs were enclosed in a small glass chamber which was not airtight, so that a continuous passage of air over the lungs was prevented, yet pressure changes for expansion and collapse of the lungs were still operative on the outer surface of the lungs. Apart from these differences the procedures adopted in the series of experiments were similar.

In the course of perfusion experiments we have observed that even in the absence of steam condensation on the lung surface the visceral pleura sometimes remains moist. It would appear that passage of fluid through the pleura takes place in these preparations. It is the exception rather than the rule for this to be observed under the conditions of our experiments and we have no explanation to offer why this should be so in some lung preparations and not in others.

The progressive changes we have observed in perfused preparations are tabulated below:—

Tidal air	−
Pulmonary arterial pressure	+ towards end of experiment
Diameter of red blood cells	− after 3 hours perfusion
Blood sedimentation rate	− if initially high
Red cell hæmatocrit	+
Hæmoglobin Fe	+
Viscosity	
blood	+ rarely decreased
plasma	+ or more rarely −
Specific gravity	
blood	+
plasma	+
Chlorides	
blood	− or 0
plasma	+ or 0

Conductivity
     blood   .   .   .   .   —
     plasma   .   .   .   .   + or −
Urea N. plasma   .   .   + or O
Total nitrogen, plasma   .   .   +
N.P.N., plasma   .   .   .   +
     + =increased;   − =decreased;   O =no significant change.

It may be said at the outset that we regard the blood changes as being mainly governed by hæmoconcentration due to increased capillary permeability. They will be discussed in the order tabulated above. The progressive reduction in tidal air may show wide differences from one experiment to another and we associate it with an initial effect of shed blood. It is an almost invariable phenomenon in perfused lungs, and our results do not enable us to implicate any one of the observed blood changes. The absence of a blood supply to the respiratory tree up to the bronchioles in lungs perfused through the pulmonary artery alone as in our preparations may be a contributory factor. On the other hand the perfused blood reaches the respiratory bronchioles and there may cause bronchiolar spasm owing to the presence of "bronchotonins" in the heparinised blood [Daly, 1938].

The terminal rise in pulmonary arterial pressure may be in part due to the increased viscosity of the blood, for an examination of Table I shows that the higher blood viscosities are associated with the higher pulmonary arterial pressures. It should be noted, however, that in two experiments (Nos. 27 and 30) in which the blood viscosity reached 167 and 163 per cent. of its initial value, no significant change in pulmonary arterial pressure occurred. Again it will be seen from fig. 3A (experiment 35) that the pulmonary pressure in each lung remained within the limits of 10–20 cm. saline during seven and one-half hours perfusion, after which time the viscosity of the blood in the two lungs reached 179 and 212 per cent. of their initial value. It would appear that an increase of 60 per cent. in blood viscosity is without significant effect upon the pulmonary arterial pressure, but higher viscosity values than this are associated with a raised pulmonary arterial pressure. Whether or no these high viscosity values are directly responsible for the raised pressure, our data are insufficient for an opinion to be expressed.

The initial high pulmonary arterial pressure in perfused lungs due to the presence of vasotonins in shed blood passes off quickly, generally within 10 minutes. The pressure immediately subsequent to this period has been taken as the initial value for the purpose of computing the percentile changes shown in Table I.

The reduction in red cell diameter requires little comment. Blood smears were not made from all the samples taken for other estimations,

but we obtained a sufficient number to show that the diameter of those cells which were spherical and non-crenated tended to diminish in size (fig. 5 and Table III). For this reason the hæmatocrit values will not show the expected agreement with the hæmoglobin iron estimations (see later).

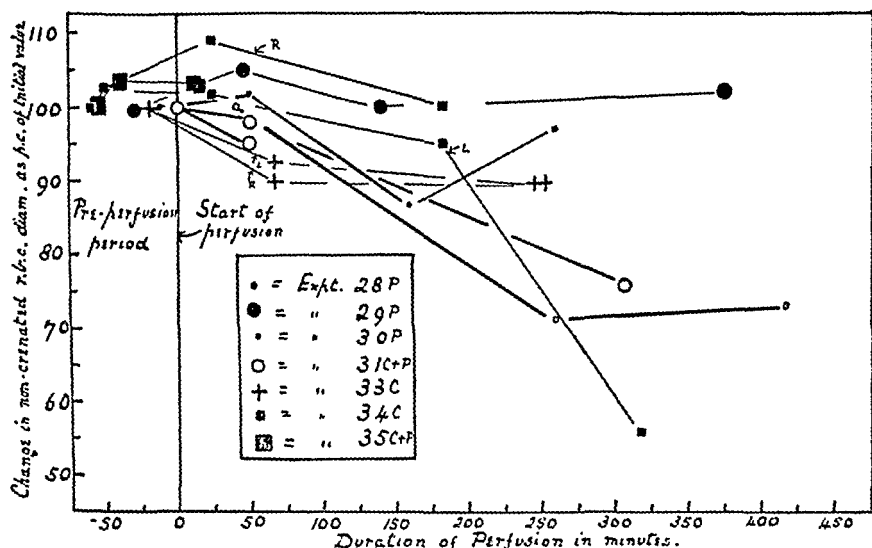With regard to blood sedimentation rate, results were somewhat

FIG. 5.—Changes in corpuscular diameter of non-crenated red blood cells due to perfusion of the blood through the lungs. The diameters are given as per cent. changes in the median value of the distribution curve. See Table III for diameter ranges.

Experiments 28, 29, and 30, gassed perfused lungs.
Experiments 31 and 35, separated perfused lungs, one control and one gassed.
Experiments 33 and 34, separated perfused lungs both used as controls.
C=control; P=phosgene.

variable, but there appeared to be a tendency for the B.S.R., if high before perfusion, to fall after a short period of perfusion.

Comparison between Hæmatocrit and Hæmoglobin Iron Values.—The blood concentration which occurs during perfusion can be evaluated from the hæmatocrit readings or the hæmoglobin iron (H.F.) measurements. It will be seen from fig. 6, which relates these two in all the experiments, that at the higher values the hæmatocrit readings are lower than would be expected from the corresponding hæmoglobin values. This may be due to destruction of red cells or to a diminution in diameter of the red cells. A small degree of hæmolysis was present in the majority of experiments which colorimetric tests in control experiments indicated was probably not more than 1 per cent. Also, as has been stated, measurement of the diameter of the red cells during perfusion revealed a progressive diminution in the diameter of the

TABLE III.—CHANGES IN NON-CRENATED RED CELL DIAMETER DUE TO
PERFUSION OF THE BLOOD THROUGH THE LUNGS.

Expt.	Sample	Time	Median	Range
28	I	Preperf.	6·8	3·9–9·0
	II	50′	6·95	3·9–8·6
	III	161′	5·95	3·5–9·0
	IV	257′	6·60	3·9–9·0
29	I	Preperf.	6·65	4·7–7·8
	II	46′	7·00	5·1–9·0
	III	139′	6·65	5·1–8·8
	IV	376′	6·85	4·9–8·2
30	I	45′	7·3	4·0–11·0
	II	259′	5·2	3·0–9·0
	III	417′	5·3	3·0–10·0
31	I	0′	6·3	3·0–9·0
	II R	55′	6·0	4·0–9·0
	II L	50′	6·2	4·0–9·0
	IV R	315′	..	..
	IV L	310′	4·8	3·0–8·0
33	I	−13′	7·6	5·0–11·0
	I a	−13′	8·4	4·0–11·0
	II R	67′	6·3	4·0–10·0
	II L	67′	7·2	4·0–10·0
	III R	250′	6·8	4·0–9·0
	III L	250′	6·8	4·0–10·0
34	I	−58′	6·8	4·0–8·0
	II	−52′	7·0	4·0–10·0
	III R	23′	7·4	5·0–10·0
	III L	23′	6·9	4·0–10·0
	IV R	181′	6·8	4·0–10·0
	IV L	181′	6·5	4·0–9·0
	V L	316′	3·8	2·0–7·0
35	I	−58′	6·4	4·0–9·0
	II	−43′	6·7	4·0–9·0
	III R	17′	6·6	4·0–9·0
	III L	17′	6·6	4·0–9·0

Perfusion start is taken as zero time.

normal shaped cells and a higher proportion of crenated and abnormally shaped cells. It is probably significant that the experiments (30 and 34) which show the greatest diminution in diameter also diverge most widely from the expected rectilinear hæmoglobin iron-hæmatocrit curve. In experiment 33 we noted exceptionally early hæmolysis which may account in part for the wide divergence from the expected values.

Specific Gravity of Blood and Plasma.—There is a tendency for both

slightly to rise.   That the red cells as well as the plasma are responsible
for the rise in s.g. of the whole blood is suggested by the results of
calculating the corpuscular s.g. from the formula

$$\text{s.g. corpuscles} = \frac{100 \,(\text{s.g. blood} - \text{s.g. plasma})}{\text{hæmatocrit}} + \text{s.g. plasma.}$$
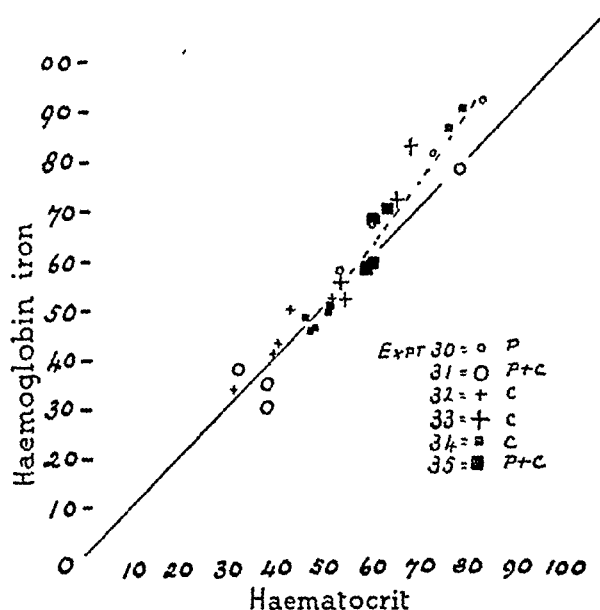
FIG. 6.—Relation between hæmoglobin iron (mg./100 c.c. blood) and hæmatocrit
(per cent. erythrocytes).

That phosgene does not determine these effects is particularly well
shown in experiment 35 (fig. 7).   The symbols in this graph correspond
to the experimental numbers shown in the other figures.

The Conductivity of Blood and Plasma.—The electrical conductivity
of plasma depends on too many factors for it to be possible to interpret
changes with certainty.   The observed changes somewhat surprisingly
show no correlation with changes in chloride concentration, and no
difference is noticeable between the behaviour of plasmas from gassed
and control experiments, such as might throw light on the mechanism
of phosgene poisoning.   The relative conductivities of blood and plasma
are a measure of the interfering effect of corpuscles, and depends on
their size, number, shape, and degree of agglutination in a manner too
complex for analysis.   Nothing in these measurements revealed any
effect attributable to phosgene.

Urea.—The percentage changes in urea concentration approxi-
mately parallel the plasma chlorides (Table I).   They call for no special

comment. Some of the N.P.N. figures of this Table show marked variations from those of the urea, which they. would be expected to parallel. These fluctuations are, however, no greater, on the whole,

$$S.g. \; corpuscles = \frac{100 \; (s.g \; blood - s.g \; plasma)}{h\alpha matocrit} + s.g. \; plasma$$
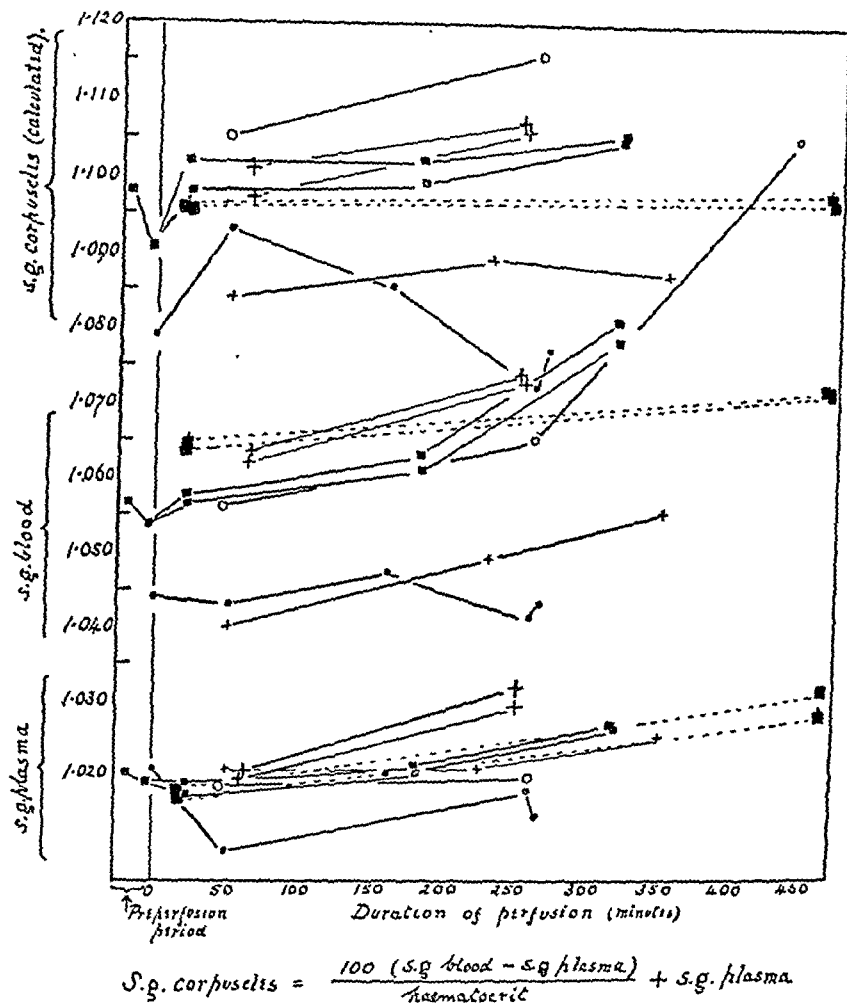
FIG. 7.—Observed changes in specific gravity of whole blood and plasma and calculated changes in specific gravity of corpuscles.

than could be explained by the low standard of accuracy of N.P.N. determinations as compared with urea.

Our results may be summed up by the statement that from the data presented none of the observed blood changes is incompatible with the view that hæmoconcentration is the sole responsible factor.   It remains to examine the conditions which led to hæmoconcentration.

THE MECHANISMS GOVERNING BLOOD CONCENTRATION IN ISOLATED PERFUSED LUNGS.

Unaccounted for Water Loss from the Perfusing Blood.—In an attempt to discover the reason for the hæmoconcentration we drew up a balance sheet of the losses from the perfusing blood and the gain in weight of the lungs on the basis that

loss in weight of the circulating blood = gain in weight of lungs
+ œdema fluid drained from lungs + expired water.

For the determination of the loss from the circulating blood column (7) Table IV), the volume of blood in the apparatus was measured (*a*) before and (*b*) after perfusion, the volume retained by the collapsed lungs during the preliminary wash-through being added to the former value (*a*), and the volume of blood drained from the inflated lungs at the end of the perfusion being added to the latter value (*b*). Inflation by excess intrapulmonary pressure squeezes out most of the lung blood. The loss of blood in the apparatus due to sampling was allowed for. The gain in weight of the lungs due to the retention of red cells, plasma, and water as separate entities or in the form of blood or œdema fluid was obtained by weighing the lungs before and after perfusion. This is given by the sum of columns (8)–(9) of Table IV. The method for calculating the proportion of blood to plasma and/or water given in columns (8) and (9) is described below in the next section. Œdema fluid when present was collected and its volume measured. Expired water obtained by condensation in the ice-cooled spiral (fig. 1) was collected and measured. Since the s.g. of blood and plasma closely approximate to unity, we have converted a volume change of 1·0 c.c. to a weight change of 1·0 g. and *vice versa* whenever necessary for the purpose of our calculations.

In 6 experiments, 3 of which comprised separated lung perfusions, there was an unaccounted water loss of 0·06–0·30 c.c./g. lung tissue/hour (see Table IV, experiments 27, 28, 32, 33, 34, 35). A feature of these experiments is the low value of the expired water vapour. If man expires 10–15 g. H_2O vapour per hour, the lungs of a 10-kg. dog would be expected to expire from 3–5 g./hr. In only one experiment was this value approached. How far this is due to a smaller active capillary bed or to the smaller ventilation in perfused lungs as compared with normal lungs, we are unable to state. The experiments demonstrate, however, that the unexplained water loss is not from the alveolar surfaces. It appeared likely that transpiration of water through the visceral pleura might be responsible. To test this we enclosed the whole lung in an airtight glass chamber, a side-connexion of which was connected to a rubber balloon. The whole apparatus was placed in the large respiratory chamber, the pressure variations of which were

TABLE IV.—ISOLATED PERFUSED LUNGS—CONTROL AND PHOSGENE-GASSED LUNGS. DETAILS OF CIRCULATING BLOOD LOST AND OF GAIN BY LUNGS OF BLOOD, PLASMA, AND/OR WATER.

Expt.		Perfusion duration, min.	Initial lung wt.	Vol. of circ. blood, c.c. Initial	Final	Lost	Lung gain (c.c.) Blood	Plasma and/or water	Exp. water, c.c.	O.F. (drained), c.c.	Sum of columns (8)–(11)	Unaccounted for water loss c.c.	c.c./g. lung/hr.	Total H.F. (mg.) in circulating blood Initial	Final	Lost	H.F. (mg.) lung gain	Lung H.F. (mg. balance)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)
27	P	280	94	810	680	130	25	10	5	0	40	90	0·20	340	335	5	11·5	+ 6·5
28	P	267	64	826	726	100	6	32	10	0	48	52	0·19	319	319	0	3·0	+ 3·0
29	P	220	83	640	560	80	8	59	2	2	71	9	0·03	320	323	−3 (gain)	8·0	+11·0
32	C	351	72	605	375	230	100	57	0	20	177	53	0·13	250	191	59	(50)	
R 33	C	263	68	440	255	185	30	79	1	0	110	75	0·25	227	202	25	(25)	
L	C	263	48	450	320	130	13	53	0	0	66	64	0·30	232	223	9	(9)	
R 34	C	408	57	421	229	192	31	97	1	31	160	32	0·08	194	166	28	(28)	
L	C	408	53	421	263	168	11	64	0	23	98	60	0·17	194	186	8	(8)	
R 35	P	423	122	599	500	99	0	36	0	0	36	63	0·06	350	350	0	(0)	
L	C	423	88	550	462	88	10	3	0	0	13	75	0·10	321	314	7	(7)	

R = right lung; L = left lung; C = control; P = phosgene-gassed lung; O.F. = oedema fluid; H.F. = haemoglobin iron.

transmitted by means of an elastic bag to the lungs for the purpose of ventilation. In this experiment (No. 29, Table IV) the lungs and glass chamber were weighed before and after the perfusion and the unaccounted for water loss found to be the lowest in the series.

This experiment, taken in conjunction with the fact that we have observed in some experiments the visceral pleura to remain moist when steam is not used in the negative pressure ventilating chamber, suggests that the visceral pleura may allow passage to significant quantities of water. Since we have also observed a flow of what appeared to be lymph in the region of the cut bronchus in perfused lungs, we are unable to say how much of the unexplained water losses is due to escape of lymph or to passage through the pleural surfaces.

Gain in Weight of Perfused Lungs.—The loss of hæmoglobin iron (H.F.) from the blood during the perfusion should equal the H.F. gain of the lungs if allowances are made for sampling and blood oozing. That such is the case within certain limits can be seen from Table IV, which shows a balance sheet of H.F. blood loss and lung gain. The mediastinal lobe was removed before the start of perfusion, weighed, and its H.F. content measured. On the assumption that the H.F. content of the mediastinal lobe represented that of the remaining lobes before perfusion, it was a simple matter to calculate the gain in H.F. content of the perfused lobes. The lungs were minced, ground in a mortar and extracted with water. The extraction fluids were always turbid, those of the perfused lung more so, weight for weight, than those of the control lobe. Turbidity tends to make the colorometric readings higher than they should be for any given H.F. concentration, and this probably accounted for the greater apparent gain in H.F. of the perfused lung than was expected from the loss of H.F. from the circulating blood. Notwithstanding, there is a fair agreement between H.F. blood loss and lung gain (Table IV, experiments 27, 28, 29). In view of these results it was assumed in the remaining experiments that the H.F. blood losses were equivalent to the gain in H.F. by the lungs.

In all the experiments there was some degree of blood hæmolysis, probably never more than 1 per cent. Neglecting this, the H.F. gain of the lungs represents red cell emboli or blood; we have no means of determining the proportions contributed by each. If the gain represents blood of a hæmatocrit value similar to that of the last perfusion sample, then the increase of lung weight due to this cause can be calculated. The figures are given in Table IV, column (8). The remaining increase in lung weight must be due to plasma and/or aqueous solutions of salts, and is presumably an indication of the permeability of the lung capillaries.

TABLE IV.—ISOLATED PERFUSED LUNGS—CONTROL AND PHOSGENE-GASSED LUNGS. DETAILS OF CIRCULATING BLOOD LOST AND OF GAIN BY LUNGS OF BLOOD, PLASMA, AND/OR WATER.

Expt.		Perfusion duration, min.	Initial lung wt.	Vol. of circ. blood, c.c.			Lung gain (c.c.)				Sum of columns (8)-(11)	Unaccounted for water loss		Total H.F. (mg.) in circulating blood			H.F. lung gain (mg.)	Lung H.F. (mg. balance)
(1)	(2)	(3)	(4)	Initial (5)	Final (6)	Lost (7)	Blood (8)	Plasma and/or water (9)	Exp. water, c.c. (10)	O.F. (drained), c.c. (11)	(12)	c.c. (13)	c.c./g. lung/hr. (14)	Initial (15)	Final (16)	Lost (17)	(18)	(19)
27	P	280	94	810	680	130	25	10	5	0	40	90	0·30	340	335	5	11·5	+ 6·5
28	P	207	64	826	726	100	6	32	10	0	48	52	0·19	319	319	0	3·0	+ 3·0
29	P	220	83	640	560	80	8	59	2	2	71	9	0·03	320	323	– 3 (gain)	8·0	+11·0
32	C	351	72	605	375	230	100	57	0	20	177	53	·0·13	250	191	59	(50)	
R 33 L	C	263	68	440	255	185	30	79	1	0	110	76	0·25	227	202	25	(25)	
R 34 L	C	263	48	450	320	130	13	53	0	0	66	64	0·30	232	223	9	(9)	
R 34 L	C	408	57	421	229	192	31	97	1	31	160	32	0·08	194	166	28	(28)	
R 35 L	C	408	53	421	263	158	11	64	0	23	98	60	0·17	194	186	8	(8)	
R 35 L	P	423	122	509	500	99	0	36	0	0	36	63	0·06	350	350	0	(0)	
	C	423	88	550	462	88	10	3	0	0	13	75	0·10	321	314	7	(7)	

R.=right lung; L.=left lung; C.=control; P.=phosgene-gassed lung; O.F.=œdema fluid; H.F.=hæmoglobin iron.

fifth hours óf perfusion and almost certainly represents the onset of œdema.

It is interesting to note that the loss of chloride to the tissues as compared with the loss to the cells is in the ratio of ten to one.

In these calculations it is assumed that the loss of red blood cells from the perfusing blood to the lungs and the change in size of the red blood cells are not significant. The graph (fig. 8) suggests that the
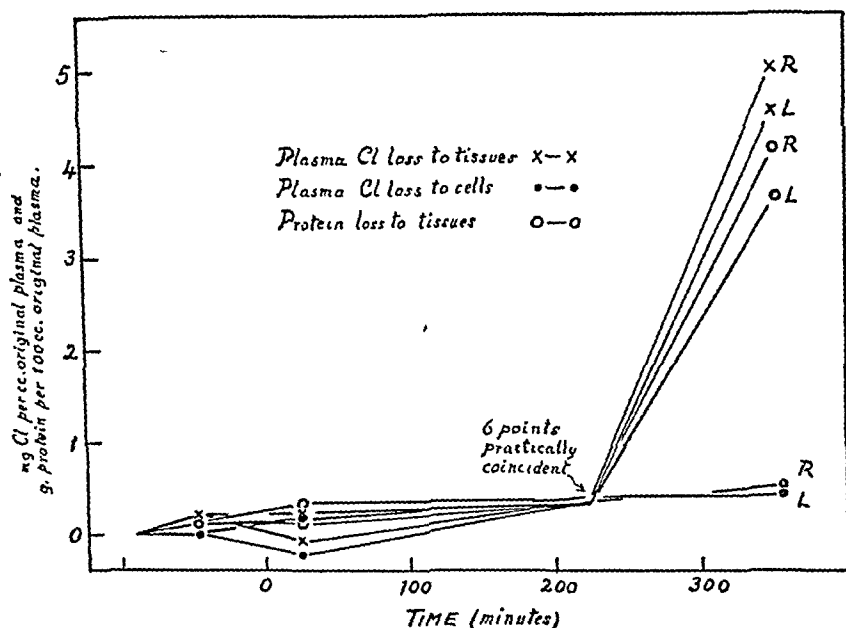
Fɪɢ. 8.—Loss of protein and of chloride to tissues, and loss of chloride to red blood cells. Perfusion started at zero time.   R =right lung; L =left lung.

determination of the hæmatocrit value and plasma chloride concentration alone will give a reliable estimate of the rate of onset of œdema. In any case it is questionable whether the calculation of the loss of chloride from the plasma to the red blood cells has significance.   If it has, it is remarkable that the diameter of the cells decreases, whereas their chloride content increases.

The rate of loss of protein from the plasma can be computed in a similar manner if it is assumed that no protein exchange takes place between plasma and red cells.   This computation has been made for experiment 34 (fig. 8).

It would be expected that as the experiment progressed and capillary permeability increased, the Cl loss from the plasma would precede and therefore finally exceed the protein loss, provided the size of molecule is the only factor determining such losses.   Reference to Table I reveals that this expectation is realized since in every experiment the

PERMEABILITY OF THE LUNG CAPILLARIES IN PERFUSED PREPARATIONS.

The progressive rise in the hæmatocrit values during perfusion indicates a loss of plasma and/or water from the blood. In the majority of experiments this loss proceeded more rapidly during the later stages of the experiment, which suggests that the permeability of the capillaries is increasing more rapidly during this period. As we have seen, the picture is complicated by the fact that part of the hæmo-concentration may have been due to formation of lymph and by transudation of water through the pleura.

Some light can be thrown upon the problem by calculating the progressive loss of chlorides and of protein from the plasma.

Let 100 c.c. of original blood contain h_0 c.c. of cells. The chloride concentration of the blood is B_0 and of the plasma R_0. Then the chloride concentration in the cells, C_0, is

$$C_0 = \frac{100}{h_0}(B_0 - R_0) + R_0.$$

If h_1, R_1, B_1, and P_1 are the corresponding values in some later sample, then

$$C_1 = \frac{100}{h_1}(B_1 - R_1) + R_1,$$

and the increase in chloride content per c.c. of cells is $C_1 - C_0$. If the cells have not changed in number or volume, then the chloride gained by h_0 c.c. of cells came from $100 - h_0$ c.c. of original plasma. Hence each c.c. of original plasma lost to the cells $\frac{h_0}{100 - h_0}(C_1 - C_0)$ mg. of chloride.

Loss to Tissues.

Each 100 c.c. of original blood has become $100\frac{h_0}{h_1}$ c.c. by loss of water. Hence chloride lost from 100 c.c. of original blood is $100\,B_0 - 100\frac{h_0}{h_1}B_1$. Since this blood contained $100 - h_0$ c.c. of plasma, the loss per c.c. of original plasma is

$$\frac{100}{100 - h_0}\left(B_0 - \frac{h_0}{h_1}B_1\right).$$

The results of these calculations for experiment 34 are graphically represented in fig. 8. Compared with the Cl losses into the tissues (or as a component of lymph), the amount entering the red cell is very small. The most rapid loss of chloride occurs between the third and

for by the blood concentration. Concentration occurs to a slight extent immediately perfusion is started and proceeds at a considerably greater rate after 2–4 hours of perfusion. We have no evidence as to the cause of the early stage of concentration, but it may be due to the loss of water by passage through the visceral plasma and in some experiments by formation of lymph. Rapid and late concentration is almost certainly due to the formation of œdema fluid. The progressive changes in the plasma chlorides on the one hand and in plasma protein on the other indicate that the chlorides leave the plasma earlier than the proteins. The composition of œdema fluid as regards the more diffusible chloride and the less diffusible protein may be determined in part by the rate of evaporation of the œdema fluid within the lungs, and therefore by the degree of lung ventilation, as well as by the rate at which the capillaries become more and more permeable to the larger molecules. These factors have to be taken into account when comparing the constituents of final samples of plasma with those of the œdema fluid.

Without prejudicing the responsibility of other factors, we wish to stress, as others have done before us, the probability that the abnormal state of the perfusion fluid accounts for the capillary damage. The perfusion fluid is heparinised blood which our experience leads us to believe more nearly approaches normal blood in its effect on the lungs than does defibrinated blood. Notwithstanding, heparinised blood on passage through the lungs quickly loses its leucocytes which are held up as clumps in the smaller blood-vessels of the lung [Bickford and Winton, 1934; Trowell, 1943]. We associate this phenomenon with the observation that in some samples of heparinised blood which has been passed through the lungs the leucocytes are found clumped together. As perfusion progresses, the proportion of irregularly shaped red cells increases and those which remain spherical and smooth in their greatest axis are reduced in diameter. That a toxic factor is responsible both for this blood picture and for the capillary damage is not unlikely. The conditions of its formation and its nature are not known. It may be identical with the so-called "vasotonin" or the "bronchotonin" substance.

Perfused Lungs Exposed to Phosgene.—With this background of our control perfused lung we have been able to test the effects of phosgene on the other lung perfused under identical conditions. The result has been that doses of phosgene sufficient to cause desquamation of the bronchial mucosa, severe bronchoconstriction, and an increase in the circulating blood volume of the lungs, exert no significant effects (as compared with the control lung) on the physico-chemical state of the blood, on the histological blood picture, on the pulmonary vascular bed, or on the rate of onset of œdema.

Various interpretations of this result occur to us, none of which has yet been tested. They may be enumerated with brief comment.

percentage increase of protein in the plasma is greater than that of the chlorides. These changes also account for the progressive reduction in the plasma Cl/protein ratio. If it is assumed that at any given moment during an experiment a solution of chloride in water equivalent to the concentration of chloride in the plasma is diffusing into the tissues and alveoli, then the concentration of chloride in the final sample of œdema fluid would be somewhat less than that in the *final* plasma samples. This follows because the chloride concentration of the plasma and therefore of any watery solution of chlorides diffusing from it progressively increases. On the other hand, any loss by evaporation of œdema fluid will tend to make the Cl concentration of the final sample higher than that in the final sample of plasma; the evaporation will also affect the concentration of protein in œdema fluid in a similar manner.

In a few experiments it was possible to estimate the protein and chloride content of the lung œdema fluid (see Table V).

TABLE V.

Expt.	Protein g./100 c.c., final samples		Chloride mg./c.c.	
	Plasma	Œdema fluid	Plasma	Œdema fluid
R 31	8·44	4·24	..	4·76
L	7·07	4·7	3·43	5·35
32	7·15	7·2	5·98	7·89
R 34	7·93	6·25	7·81	7·75
L	8·10	6·28	8·36	8·11

The chloride of the œdema fluid is higher than that of the plasma in two of the experiments, which may be in part due to evaporation. The protein concentrations of the œdema fluid are (with one exception) lower than that of the plasma in spite of œdema fluid evaporation. We attribute this to the predominating effect of diffusion of protein late in the experiment when it diffuses into already formed protein-free œdema fluid. We realise that where diffusion through membranes is concerned, concentrations of chloride are more appropriately expressed per 100 g. of water than per 100 c.c. of plasma, but in practice such correction does not affect the conclusions given above.

DISCUSSION AND SUMMARY.

Control Perfused Lungs.—The biochemical changes in the blood which is perfusing isolated lungs can for the most part be accounted

THE PHYSIOLOGICAL EFFECTS AND FATE OF CYANOGEN CHLORIDE. By W. N. ALDRIDGE and C. LOVATT EVANS.

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CYANOGEN chloride, a colourless volatile liquid obtained by direct chlorination of hydrocyanic acid, has B.P. 13° C., F.P. – 6° C., and at normal temperature and pressure a vapour density of 2·14 (air = 1); if impure, it readily polymerises to cyanuric chloride, but is relatively stable when pure. It has a pungent, peppery smell, with a background of bitter almonds, and is extremely irritating to breathe. It is soluble in most organic solvents and up to 7 or 8 per cent. in water at 8° C., in which at neutral or acid reactions it is fairly stable at room or body temperatures. In alkaline solution, it is rapidly hydrolysed, first to cyanic acid and HCl:

$$CNCl + H_2O = HCNO + HCl,$$
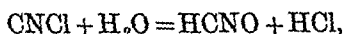
which is later slowly converted to CO_2 and ammonium chloride:

$$HCNO + HCl + H_2O = NH_4Cl + CO_2.$$

Cyanogen chloride was used to a small extent as a war gas in the war of 1914–18, but no detailed study of its mode of action seems to have been made.

GENERAL EFFECTS.

When conscious animals or men are exposed to atmospheres containing cyanogen chloride, in a concentration of, say, 100 mg./cu.m., they at once experience resistance in, and irritation of, the respiratory passages, and smarting of the conjunctiva, with severe blepharospasm and lachrymation. Some species, *e.g.* dogs and rabbits, hold the breath for a time. This must be a conscious reaction, as it is not seen under anæsthesia. A certain amount of adaptation to the gas occurs after a minute or two with concentrations up to 100 mg./cu.m., so that the initial symptoms decline in severity. Sensitive species, such as dogs, will nevertheless in time suffer general systemic effects: even with concentrations as low as 50 mg./cu.m., easily supportable by man, we have seen death of individual dogs occur after two hours exposure, although other dogs simultaneously exposed were but little affected; while others, though severely convulsed, even survived exposures for a further two hours at 100–120 mg./cu.m.

VOL. XXXIII, NO. 4.—1946.   241   17

1. It might be argued that in an experiment of 7–8 hours the full effects of even very large doses of phosgene on the lungs have not time to become manifested, for in our experience of phosgene-gassed living dogs there is a latent period of 8–10 hours after gassing before clinical signs of œdema appear. This indeed may be the correct interpretation of our results. If so, it is clear that the conditions under which isolated lungs are perfused cause œdema before phosgene œdema has time to appear, and thus isolated perfused lungs are unsuitable for testing the effects of phosgene administration on lung tissues. We are not, however, inclined to accept this interpretation without further evidence.

2. That the severe bronchoconstriction without a compensatory increased respiratory effort effectively prevents high concentrations of phosgene from having contact with the alveoli for any significant length of time.

3. That the lung tissues and capillaries in perfused lungs differ in respect of their phosgene reactivity from those of normal lungs. How far the absence of functionally active nerves to isolated lungs may be responsible for this difference requires examination.

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3·7 g./cu.m., it only fell after 5 minutes to 2·8 g./cu.m.   Since exposures were only as a rule of about one or two minutes duration, the decline, of not more than 0·2 g./cu.m./min., could be disregarded for present purposes.   The procedures employed for recording were those common in physiological laboratories, or simple improvisations of them.

I. Effect on the Circulation.

Arterial Pressure.—The usual effect on the anæsthetised animal with intact nervous system was a transitory rise, followed by a fall (fig. 1); occasionally there was a rise superimposed on a ˉfall, and in the decapitate cat the only result was a fall (figs. 7, 14).   A fall was also often the only effect of doses following a first heavy dose.   There is a general similarity between the effects of cyanogen chloride and those of HCN [Lovatt Evans, 1919], as is illustrated by figs. 1 and 2. The effects on arterial pressure are the resultants of contending factors, some making for a rise, some for fall, as will be shown later.   When the vasomotor centre has been eliminated by decapitation, or as an after-effect of a large initial dose of cyanogen chloride, so that there is lowering of the peripheral resistance and of vascular tone, cyanogen chloride produces a fall in arterial pressure.   Early reflex effects otherwise tend to produce a rise, and so does an early increase of cardiac output.   The final fall to low levels is due to the combined action of extensive vascular relaxation and lowered cardiac output.

The heart rate is ultimately slowed, but sometimes there may first be an early slowing, succeeded by some acceleration.   These early effects are mainly due to reflexes from the carotid bodies, and perhaps from other chemo-receptors, since after combined denervation of the carotid sinus and vagus section they give place to the ingravescent slowing which is the usual direct effect of the substance on the heart.

The slowing of the heart which appears at a later stage of the poisoning is of two types.   The first type appears suddenly, and may with small doses vanish again quite abruptly: when this happens after double vagotomy (*e.g.* in figs. 4, B; 9, B) it would undoubtedly be due to transient interference with conductivity in the bundle of His, and is a phenomenon seen also in cyanide poisoning, and as a result of oxygen lack in general.   The other type of slowing may be regarded as due to a more generalised effect of the poison on the myocardium, including the pacemaker and conducting structures: ultimately the rate becomes very slow, and the force of the beat greatly weakened (figs. 3, C; 4, C; 13, D.)   As in other forms of oxygen lack, however, an early effect on the myocardium, as seen in the saline-perfused heart, is at first negligible.

General venous pressure, as measured in the great veins, does not rise with the arterial pressure, but when the latter begins to fall, usually

Vomiting and deep respirations are early symptoms of the poisoning, and loss of consciousness soon follows. Convulsions, when they occur, are often of extreme violence, and may occur, with intermissions, for an hour or two after removal from the atmosphere. The fits are alternated with quiet periods, with very deep breathing, general exhaustion, continued loss of consciousness and of reflexes, widely dilated pupils, and bright scarlet mucous surfaces. The intermittent fits usually commence with a tonic extensor spasm, followed by explosions of vigorous progression-movements, which gradually die down again. Despite the severity and duration of these symptoms, recovery is often complete, sometimes within 30 minutes. When complete recovery does not follow, there is either death or disablement. Death is often preceded by copious, and occasionally sanguineous lung œdema: respiration fails long before the circulation. The disablement is referred to later.

Post-mortem findings reveal little beyond inflammation of the upper respiratory tract, some lung œdema, and dilated right ventricle with engorgement of the venous system. Some persons claim to recognise the odour of cyanogen chloride in the brain of animals killed by it.

The experimental work described below has necessarily been carried out on anæsthetised, or on decapitated or decerebrated animals. There is no reason to suppose, however, that, apart from cortical responses, to which reference is made in the proper place, the anæsthetic has introduced any qualitative change into the picture.

METHODS.

Nembutal was the usual anæsthetic. Decapitations and decerebrations were performed under ether or C–E. The cyanogen chloride was, in some instances, administered by the intravenous injection of assayed solutions in 0·9 per cent. NaCl solution, but more usually by inhalation. For the latter purpose, a carboy of 36 l. capacity was used to contain the air-gas mixture, a common concentration being 3 g./cu.m. Administration of this was effected in one of two ways: (a) by the use of delicate respiratory valves connected with a trachea tube, the animal was supplied, through a short wide tube and 3-way tap, at will either with air or with the gas mixture. The fall in concentration of gas as the contents of the carboy were slowly displaced by entering air was negligible over short periods. (b) When administration by artificial respiration was essential, the gas was drawn from the carboy, and delivered to a T-trachea tube by means of a Brodie pump, the free outlet of the trachea tube being provided with an absorbent container. By this method, when cats or rabbits were used, the rate of fall of concentration in the carboy was more rapid than in method (a), but was still not such as to vitiate results: e.g. starting at

runs parallel with it in the earlier stages of the action (fig. 3, A). It would seem, then, that the heart is, at these stages, able to cope easily, with the venous return, and it is probable that the usually augmented pulmonary ventilation considerably facilitates this. At the later
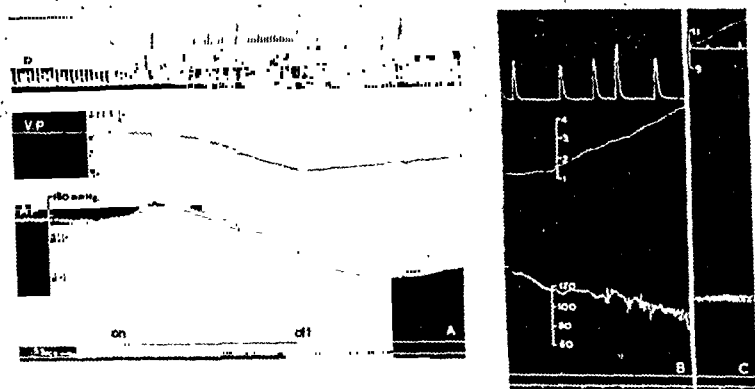

Fig. 3.—Cat. Nembutal. Respirations (R), Venous pressure in r. ext. jugular v. (V.P.) and carotid artery pressure (A.P.). A, inhalation through valves of CNCl, 3 g./m.³ for 1 min. B, C, later effects of prolonged administration, at about 1·5 min. and 3 min. respectively.
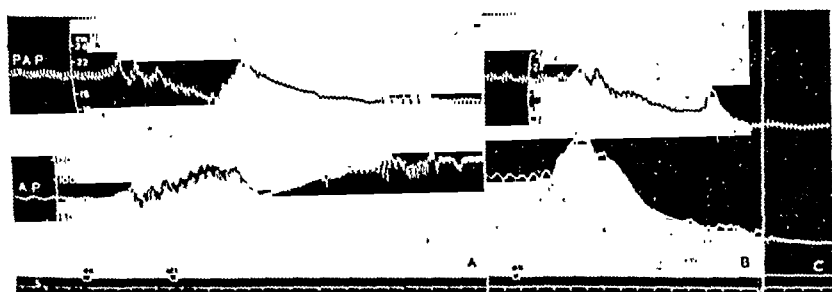
Fig. 4.—Rabbit. Nembutal: artificial respiration. Pulmonary artery pressure (P.A.P.) in cm. H₂O and carotid artery pressure (A.P.) in mm. Hg. A, administration for 35 sec. of CNCl, 3 g./m.³. B, 10 min. later: vagi divided: administration of CNCl, 3 g./m.³ for 100 sec. C, 40 sec. after end of dose.

stages, when respiration is failing, and the heart already weakened, the venous pressure rises rapidly (fig. 3, B, C), and at this point inspection of the veins shows them to be tensely gorged with blood: this is the condition at death.

Pulmonary artery pressure (which was measured in the opened chest) at first moves reciprocally to the general arterial pressure (fig. 4, A), though the movements (in terms of H₂O) are slight in any case, and give no hint of pulmonary resistance. The same is true at later stages and also after vagotomy (fig. 4, B, C), and we may assume that if any resistance to the pulmonary circulation occurs at all, it can only be
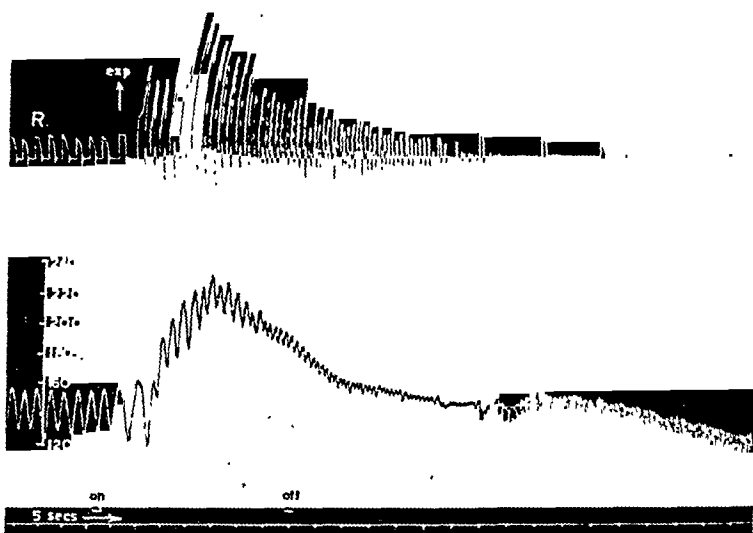
FIG. 1.—Cat. Nembutal: valve breathing: administration of CNCl, 3 g./m.³ till death. R, respiration, expiration only. M, record of general muscular movements, which here were very slight. A.P., arterial pressure.

In all tracings writing points are vertically lined unless otherwise shown.
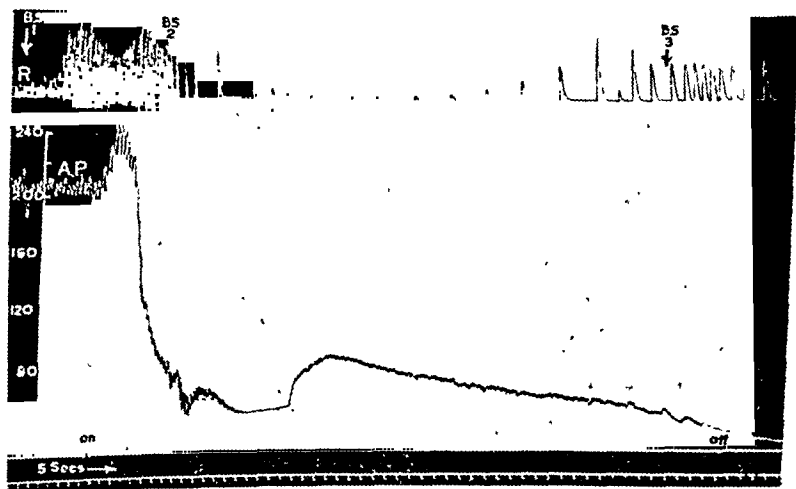
FIG. 2.—Cat. 3·8 kg. Nembutal: breathing by valves. Inhalation of HCN, approx. 1 g./m.³ (which is chemically equivalent to 75 per cent. of the CNCl, of 3 g./m.³ shown in fig. 1), for 265 sec. R, respirations. A.P., arterial pressure. Blood samples (B.S.) taken before Z, and at Z +35,240, and 375 sec.

the rising arterial pressure, as often happens in this region.   It can be concluded from this that splanchnic area changes in all probability do not contribute predominantly to the early rise in arterial pressure. This is confirmed by the fact that in the eviscerated animal (from which the whole abdominal alimentary tract and spleen have been removed, and the liver circulation much diminished) the rise of arterial pressure is particularly well shown (fig. 6).
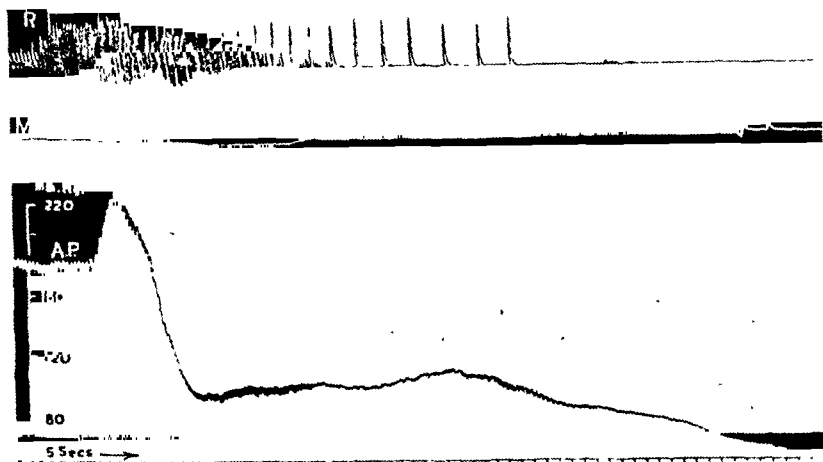
FIG. 6.—Eviscerated Cat.   Respirations and arterial pressure.   Inhalation by valves of CNCl, 3 g./m.³ for 40 sec.   Partial recovery occurred in 15 min.

Circulation Volume.—One experiment, on a dog, was carried out for us by Dr. H. D. Bruner, using a bubble stromuhr devised by Schmidt. This, intercalated in the femoral artery, measured the flow through the limb.   Intra-arterial injection of 1 mg./kg. of CNCl caused, at first, considerable increase in blood flow (100 to 140 ml./min.), presumably owing to a direct dilator action of the drug;   this was followed by a transitory fall (to 80 ml./min.) parallel with falling arterial pressure. Intravenous injections showed an increased flow with small doses (1 mg./kg., 95 ml./min. to 110 ml./min.);   with larger doses (3 mg./kg.) the general trend was a fall (170 ml./min. to 30 ml./min.) as the arterial pressure fell, and the heart failed.

The Cardiac Output.—The output of the heart was roughly assessed by recording the pulse rate and heart volume by means of a Henderson cardiometer (since other methods were not available, or were inapplicable).   In the records (figs. 7, 8) systole is downwards and diastole upwards.

It will be seen that there is after a time a considerable increase in cardiac output, the stroke volume being augmented more than the heart rate is reduced.   This increase can hardly be regarded as reflex

at pre-terminal stages, when the left ventricle and auricle fail to empty.

This possible stagnation of flow has never been actually seen, which is a point of some importance, since its non-occurrence or very late appearance reduces the possibility that lung œdema, when it occurs, might be due to the mechanical cause of raised pulmonary pressure, rather than to damage to the alveolar membrane.

Taken together then, the arterial, venous, and pulmonary artery pressures show that up to a late stage the heart is functioning well, and clearing the venous system and pulmonary circuit adequately. We may also infer that when the heart does fail, the two ventricles fall out together.

Vascular Tone.—Alterations in the peripheral circulatory resistance furnish only a partial explanation of the changes in arterial blood
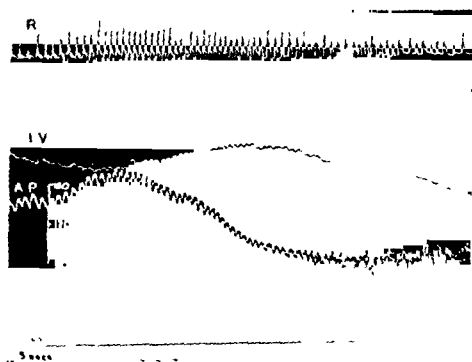
FIG. 5.—Cat. Nembutal: respiration through valves (expiration upwards). Respiration (R) volume of intestinal loop (I.V.), and arterial pressure (A.P.). Inhalation of CNCl, 3 g./m.³.

pressure. Thus, the skin vessels, as judged by plethysmographic tracings of the leg volume at first show slight active constriction with rise of arterial pressure, though afterwards, with falling A.P., the continuing constriction is largely passive in character. That it is the skin vessels that are here principally concerned is shown by the fact that the skinned limb shows no consistent change in volume. It may be that, as is the case in cyanide poisoning, a discharge of adrenaline into the circulation accompanies the early phase of the poisoning, and so contributes to the general vasoconstriction, but if this is so, the effects are not conspicuous.

As a sample of the splanchnic area, the volume of an intestinal loop was studied. This sometimes showed active constriction as the blood pressure rose, though it was never very powerful (fig. 5); *later*, or in the decapitate animal immediately, there was an active dilatation (fig. 5), and at other times the weak early constriction gave way before

off from 204 c.c./min. to 8 c.c./min. just before death, the rate being reduced from 160 to 22 per min., and the stroke volume from about 1·3 c.c. to about 0·3 c.c. per ventricle. The diastolic volume of the heart was at the same time enormously increased (by about 10–11 c.c.) as the power to empty got progressively less and less.

There is little doubt that the increased cardiac output, in presence of maintained arterial tone, provides the main explanation of the early rise of arterial pressure in the animal with intact nervous control. After decapitation, or spinal transection (figs. 7, 8, 14), the initial rise of blood pressure was not seen, although at first the rise in cardiac output occurred; this was probably due to the fact that the vaso-constrictor reaction was replaced by a direct relaxing effect of the poison on the peripheral vasculature.

II. Effect on Respiration.

The general course of the effects on respiration, and their relation to the circulatory changes, when a rapidly fatal dose of cyanogen chloride is inhaled is illustrated by fig. 1. As with conscious animals, almost immediately the gas mixture reaches the respiratory passages violent and rapid coughing sets in; this soon subsides, and, for a variable period, is succeeded by deep, and sometimes rapid, breathing (fig. 1); this then becomes progressively reduced in depth and frequency, and finally ceases, often after a spell of irregular, decelerating, gasping breaths. The circulation continues to function for some minutes after respiration has failed. After long pause in the breathing, deep gasping respirations may recommence, and, if the administration has then ceased, may be followed by recovery, in which case breathing generally remains slow and deep for a long period, or else the animal may succumb after all, pulmonary œdema being a frequent termination.

After an animal has made a precarious recovery, the response to a further dose is modified—coughing is now reduced, or absent, probably because of damage to the sensory surfaces, and the increase of ventilation is much less evident, probably because of damage to the respiratory centre or to the various chemo-receptors.

It may easily be shown that both the coughing and the raised ventilation are mainly of reflex origin; section of the vagi abolishes the coughing, but leaves the augmented breathing well in evidence (fig. 9, A, B); if vagotomy is followed by denervation of the carotid sinuses, administration of cyanogen chloride leads, at first, not to increase but to inhibition of breathing (fig. 9, C). This effect is also seen with cyanide, or with oxygen deprivation, and proves that most of the usually augmented breathing on inhalation of CNCl is reflexogenic and not centrogenic in origin, the excitability of the respiratory centre, in fact, being depressed. In some cases, after elimination of the chemo-

in origin, since it occurred in a cat with spinal transection at the 2nd cervical (fig. 7). The increase in one experiment, for instance, was from 166 c.c./min. to 274 c.c./min., although there had been a slight fall in
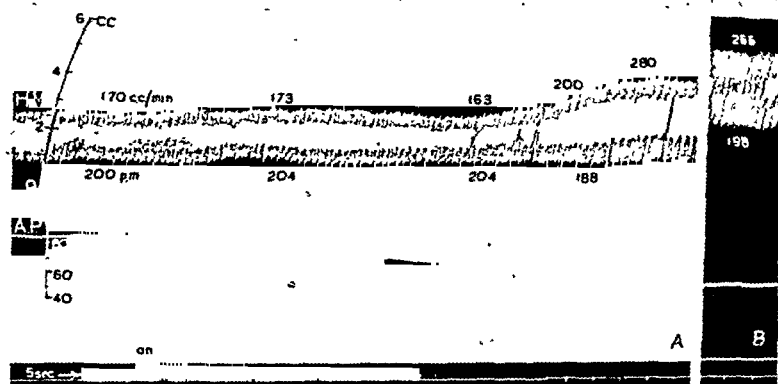

FIG. 7.—Cat. Spinal transection at C2. Cardiometer tracing and carotid pressure. Figures above heart volume show cardiac outputs in c.c./min.: figures below show heart rates. At the signal, CNCl, 4 g./m.³, administered by artificial respiration, and continued for 3 min. B, shows state 1 min. after end of dosage.
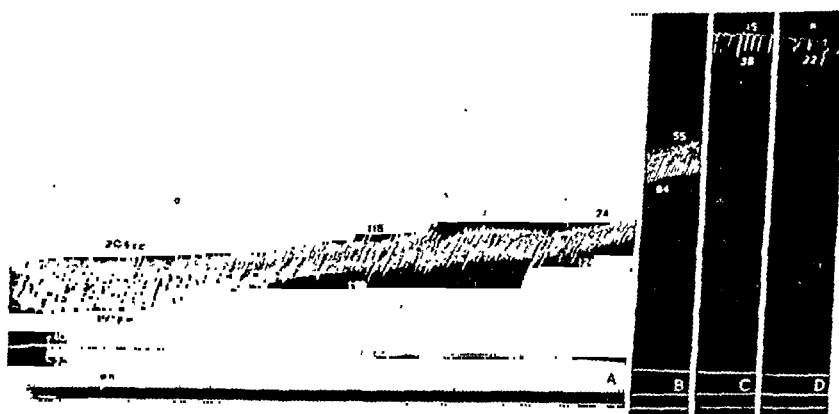
FIG. 8.—Same as fig. 7, 1·5 hours later. The cardiac output is still high. At signal, CNCl given, 8 g./m.³, until death. B, C, and D show state at 200, 260, and 340 seconds from start.

pulse rate from 208 to 196; later, as the pulse got slower (158), the output per minute fell off again to 237 c.c., the output per beat remaining about the same (about 1·4–1·5 c.c.). The increased output is accompanied by an increased diastolic volume, as is usual, and is a phenomenon often seen in the early stages of oxygen deprivation.

At later stages of the poisoning, the output continually falls, to reach very low levels and becomes enormously dilated, and finally flickers out (fig. 8). In that experiment it will be seen that the cardiac output fell

was an evident resistance, and it was reversible ; the latter fact probably rules out œdema as one of the causes of the resistance.

From these experiments it is inferred that the course of events on inhalation of cyanogen chloride is somewhat as follows: first, the upper respiratory passages are stimulated, with resultant coughing; then, as toxic substances circulate in the blood, a powerful stimulation of the carotid bodies, aortic bodies, and any other chemo-receptors ensues, with consequent hyperpnœa; the effect on the respiratory centre, like that of oxygen lack, is essentially a paralysing one, and later, a paralysing
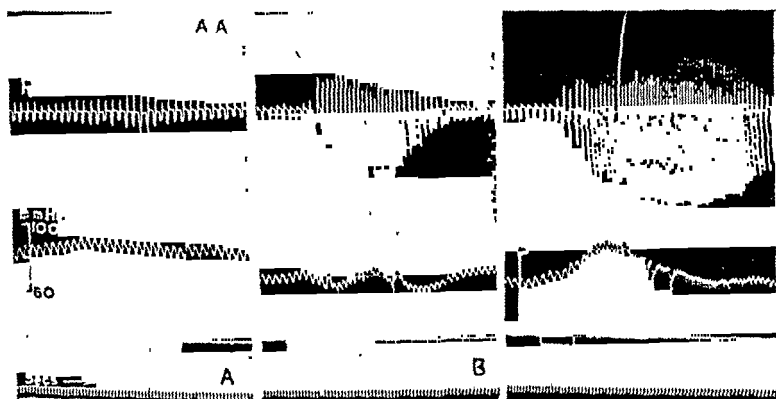
FIG. 10.—Rabbit. Nembutal and Intocostrin *: artificial respiration: record of lung volume and arterial pressure. A, administration of CNCl, 3 g./cu.m., causes intense broncho-constriction. B, 15 min. later. C, stimulation of 1. vagus.

effect is exerted on the chemo-receptors too, so that we are left with a failing respiratory centre, the activity of which is confined to the production of gasps, and finally even this fails. The onset of respiratory failure is no doubt accelerated by the preliminary stage of hyperventilation, with washing out of CO_2, followed by failure of the stimuli from the chemo-receptors.

The effect of the increased breathing on the circulation has been referred to above: these movements, in the normal closed chest, together with the effect of broncho-constriction, would probably lead to an increase of cardiac output, even greater than that which we were able to demonstrate in the animal with open thorax.

III. FATE OF CYANOGEN CHLORIDE IN THE BLOOD.

Stability of CNCl in Aqueous Solution.—Up to pH 8·0 CNCl is fairly stable in aqueous solution, as the following experiment shows: about 7 μg./ml. of cyanogen chloride was used, in a solution of 1 per cent. KH_2PO_4 brought to the required pH by NaOH titration by glass

* A curari substitute.

receptors, cyanogen chloride administration causes merely a reduction instead of complete inhibition of breathing. In either case, the period of reduction or inhibition of respiration is usually followed by a fugitive augmentation of variable magnitude; this is, we think, attributable to an effect of the mounting CO_2, upon the centre. Decerebration at the level of the superior colliculi, with vagi left intact, also seems to abolish the cough reflex; no explanation can be offered for this at present, as it is difficult to see what important afferents could have been eliminated, and we are left with the supposition that the cough
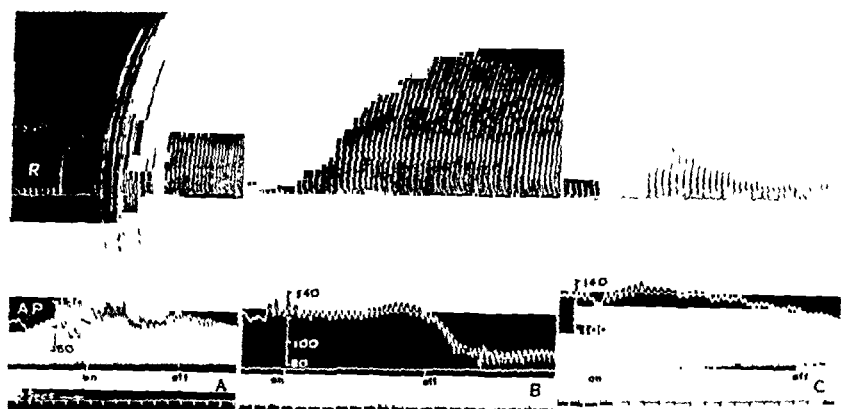
Fig. 9.—Rabbit. Nembutal. Inhalation of CNCl, 2 g./cu.m. A, before further operation. B, after section of both vagi. C, after denervation of carotid sinuses.

must be influenced in some way by centres lying above the level of the pons.

Effect on Calibre of Air Passages.—In these experiments the chest was opened, and artificial respiration carried out by rhythmical blasts from a Brodie pump, with free escape from a T-trachea tube; the volume of the lungs was recorded, each inflation causing an upward movement on the tracing. On administration of the CNCl there followed within a minute a slowly gathering obstruction to collapse of the lung after each inflation, so that the lung remained blown out. After cessation of the administration the effect slowly disappeared (fig. 10, A, B). As a check on the method it was demonstrated that stimulation of the vagus likewise caused broncho-constriction (fig. 10, C). After cyanogen chloride administration the lungs were not only inflated but always looked extremely vascular and of a scarlet colour.

With the method employed one cannot be certain that the resistance to inflation of the lungs after cyanogen chloride is wholly due to constriction of the bronchiolar musculature; part of it might have been due to vascular engorgement, or to œdema, but whatever the cause there

ently not so rapid as to make the toxicity appreciably different whether administered by inhalation or by injection.

Behaviour in Body when Inhaled.—The above considerations lead to the question whether, in fact, either CNCl or a potent derivative of it demonstrably persists in blood for an interval of time adequate to allow it to produce effects.   It will be seen from fig. 11 that the injection of the CNCl-blood mixture is *not entirely without effect, and* if the dose of cyanogen chloride added be large enough, quite a considerable effect can be produced, and this shows no diminution on further
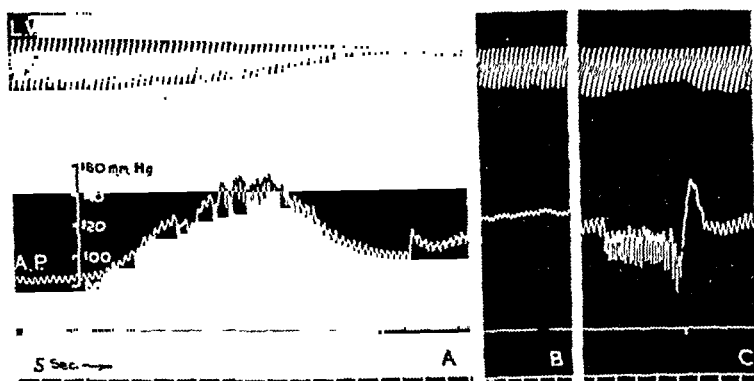
FIG. 11.—Rabbit. Nembutal. A, intravenous injection of 0·8 mg. CNCl, in 4 c.c. blood (heparinized). B, intravenous injection into R. ext. jug. of 0·8 mg. CNCl in 4 c.c. saline. C, (on right) intravenous injection of equivalent amount of HCN (0·35 mg.) in 0·35 c.c.

keeping of the mixture.   To decide whether the effects of the substance were, at least in part, blood-borne, recourse was had to cross-circulation experiments.   Two cats were used, side by side, and a carotid–carotid cross-circulation was set up between them, so that blood from cat 1 continually passed to the peripheral end of the carotid of cat 2 (and so to his carotid body), while blood flowed *vice versa* from cat 2 to cat 1. Cat 1, breathing by valves, was given the inhalation of CNCl, with the usual effects on his blood pressure and respiration; after a brief delay, similar, though milder effects, were produced on cat 2 (fig. 12, A). Next, the crossed circulation was interrupted for a time, and cat 1 again given an inhalation of CNCl for a period, and then, about 5 seconds after the end of that period, the crossed circulation was re-established. Again cat 2 showed unmistakable effects on respiration, which is always the more sensitive reaction; effects on arterial pressure were equivocal.

   This experiment proves beyond doubt that, despite the rapid disappearance of CNCl from blood, effects of it are at least in part capable of being blood-borne, either because some CNCl itself reaches the tissues or because some other toxic product is formed from it, and does so.

   Slightly to anticipate what is to follow, it may be stated that, whether

electrode. Samples were removed at intervals for analysis by omitting the bromination stage in the method described by Aldridge for HCN [Aldridge, 1944, 1945]. At room temperature (18–20° C.):

pH.	$t.$	CNCl μg./ml.	$k \times 10^3$ (hr.).
4·47	0	7·33	..
	2 hr. 22 min.	7·41	..
	42 hr.	5·71	5·9
6·94	0	7·06	..
	2 hr. 18 min.	6·70	..
	42 hr.	1·85	31·8
7·52	0	7·17	..
	33 min.	6·86	..
	2 hr. 18 min.	6·30	56·2
	42 hr.	0·91	49·0
8·0	0	7·17	..
	32 min.	6·86	..
	2 hr. 17 min.	6·11	69·8
	42 hr.	0·39	69·8

Stability in Blood.—At blood pH (around 7·4) therefore, change in aqueous solution is slow, and it might be expected to be similarly stable in the presence of blood. This, however, is not the case, as will be seen by the next experiment, and, later, by direct chemical proof (which is the order in which the experiments were made). In fig. 11, A, is shown the effect of injection of 0·8 mg. of CNCl, which a few seconds before had been added to 4 ml. of the animal's own blood, heparinised to prevent clotting, and in fig. 11, B, the effect on the same animal of the same amount of CNCl injected in solution in 0·9 per cent. of NaCl solution. For comparison, fig. 11, C, shows the effect of the chemically equivalent amount of HCN (0·35 mg.). The results prove, first, the arresting fact that CNCl is rapidly altered by blood, and second, that an equivalent amount of HCN is much more effective. Destruction of CNCl by blood cannot be instantaneous, or injection would be of no effect anyway, but it is undoubtedly only a matter of seconds at 37° C. The time of transit from the site of injection (external jugular vein in fig. 11) to the carotid sinus would, in a small animal like a rabbit, be of the order of a second only. We thought that by injecting the substance into a more peripheral vein, such as the femoral, and so adding to its journey a further time of transit it might perhaps be possible to show that a smaller effect was produced than when injected into the jugular, but, in fact, found no measurable difference in the effects. Experiments *in vitro*, however, showed that the rate of destruction in blood was quite high, but appar-

CNCl it was, in fact, possible to detect HCN in the animal's blood. The HCN was estimated by Aldridge's method. First, a pre-dosage sample of blood was taken from the nembutalised animal. Then the animal
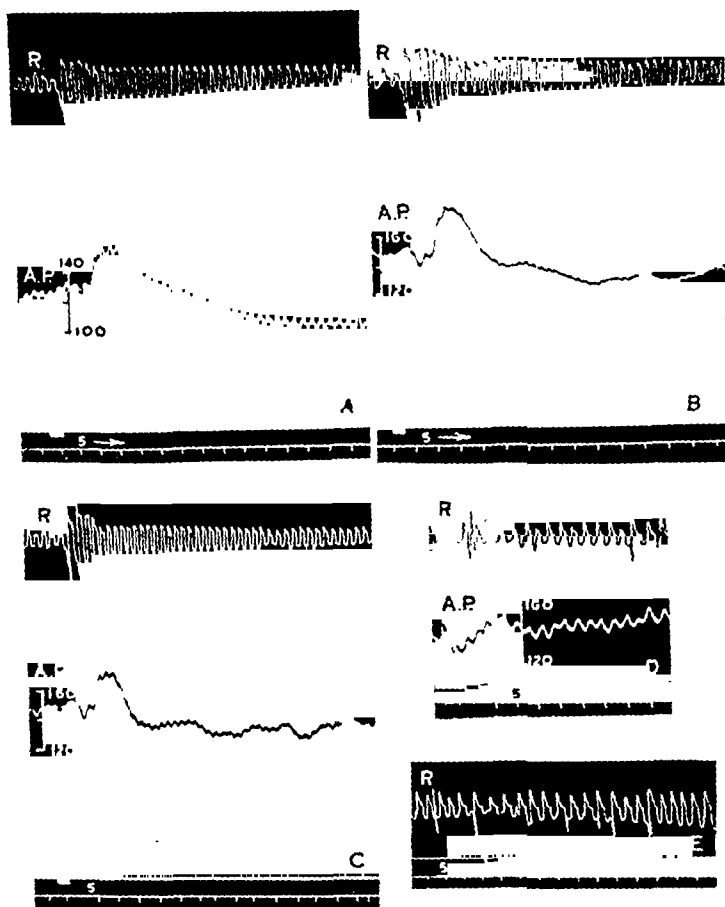

FIG. 13.—Cat. Nembutal: vagi intact: administration by intravenous injection. A, 0·95 mg. CNCl in 3 c.c. into l. femoral vein. B, 0·144 mg. HCN (= 35 per cent. of amount equivalent to 0·95 mg. CNCl) as KCN, into l. femoral vein. C, 0·122 mg. HCN (equal to 30 per cent. equivalent of 0·95 mg. CNCl) into l. femoral vein. D, 0·95 mg. CNCl into portal (splenic) vein branch. E, 1·9 mg. CNCl into portal vein branch (no pressure record). R., respiration. A.P., arterial pressure.

inhaled till the end of the experiment a chosen concentration (3 g./cu.m.) of CNCl through the respiratory valves, records of arterial pressure and respiration being taken throughout; blood samples for analysis were collected from the carotid artery at intervals. The tracing resembled that of fig. 1 so closely that it need not be reproduced. The results were as given overleaf.

or not *any* of the actions of CNCl (beyond those on surfaces directly exposed to it) may be due in part to the substance itself, it is beyond doubt that much of its effect is due to its very rapid conversion into HCN by the blood. The conversion is not quantitative, but generally seems to represent the change of about 30 per cent. of the substance into HCN. This must be discussed in detail later, since it is unexpected from the chemical point of view.
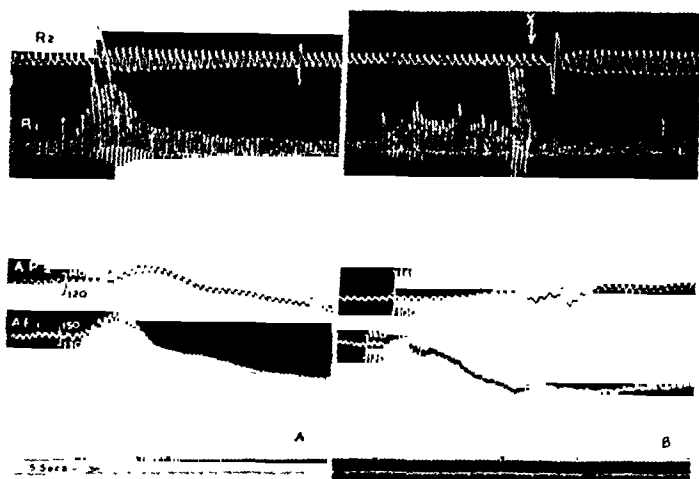

Fig. 12.—*Two cats. Nembutal. Heparin: cross-circulation established, carotid to carotid. Cat 1, breathing by valves, received the CNCl, 3 g./m.³, between signals. Cat 2, breathing by T-trachea tube, received no gas by lungs. A.P. 1, femoral artery pressure of cat 1. A.P. 2, femoral artery pressure of cat 2. R.1, respiration cat 1 (expiration). R.2, respiration (insp. and exp.) of cat 2. In tracing A the cross-circulation was in action from start. In tracing B the cross-circulation was cut off to begin, and was opened again at arrow, just after administration of CNCl stopped.*

Comparison of the effects of known doses of CNCl and of HCN given intravenously, on arterial pressure and on respiration (particularly the latter), show that the CNCl exerts an effect which is roughly 30 per cent. of that of the chemically equivalent amount of HCN. Thus (fig. 13, A, B, C) 0·95 mg. of CNCl produces about the same effect as 0·12 mg. of HCN. It is perhaps worth recording also, that when injected into the portal vein, so that it goes first through the liver before reaching the general circulation, CNCl produces but slight or no effects (fig. 13, D, E). Since the substance is all destroyed, and largely by conversion into HCN, this may mean that that substance is detoxicated in the liver; but although we have obtained direct evidence from liver perfusion and other experiments that this is so, we did not find the detoxification to occur very rapidly in the liver.

Further to test the matter, and to seek confirmation of the *in vitro* experiments, attempt was made to find whether during inhalation of

IV. Effect on the Central Nervous System.

In considering the effect of CNCl on the central nervous system,
it must be borne in mind that most of the experiments here described
have necessarily been performed on anæsthetised animals.   Consequently
convulsions, and the vomiting, such prominent features when intact
mals are used, are commonly in abeyance.   Fig. 6, which is typical,
ws that under these conditions general muscular movement is, on
whole, slight.   Sometimes there are pre-mortal spasms when the
æsthesia is light, and a much reduced type of spasm may occasionally
en in the spinal or decerebrate animal, from which the volatile
used for the initial operation has been blown off, but these
he tempestuous character of the convulsions seen in the
hetised animal.   It may be inferred from this that the
ainly of cortical origin, and that the remaining motor
subsidiary part in their production.
s of CNCl are often to produce facilitation or
r and spinal centres, with resulting outbursts
oxygen lack in general, there occurs a sym-
uch symptoms as pupil dilatation, vaso-
dio-inhibition of central origin, as well as
centre by way of the outlying reflexogenic
state of excitation gives way to one of
reads from the higher to the lower centres.
n early, the conjunctival reflex disappears,
ressed and probably the chemo-receptors
, and spinal reflexes are abolished.   This
illustrated by fig. 14 in which the knee-
aps on the patellar tendon with an
scribed by Schweitzer and Wright
e respiration fails, but returns
a small dose.   Convulsions, when
tage than that of the loss of the
assume that the latter is to be
rather than to paralysis of

h as leads to protracted
itation is only feasible
the central nervous
damage is to be
dose, the effects
thal exposures,
possibility of
indeed, is the
.   We have
18

Time from zero.	A.P., mm.Hg.	Relative respiratory ventilation.	HCN content of arterial blood, μg./ml.
Pre-zero . .	140	44	0
30 sec. . .	180	260	..
1 min. . .	134	106	3·0
3·5 min. . .	88	15	4·1
6 min. . .	54	8	4·4

In no case was any CNCl found in the blood. Thiocyanate of the blood was raised to about double its original level, but could not be accurately estimated in whole blood, and plasma was not available in sufficient amount for analysis. These results prove that when CNCl is being inhaled in concentrations that, though by no means high, would soon prove lethal, HCN almost immediately appears in the arterial blood, and so would quickly be delivered all over the body. In the absence of any known experimental data as to what concentrations of HCN in the blood might be expected to prove lethal, a parallel experiment was performed on a cat inhaling an HCN-air mixture of ultimately lethal composition. The concentration chosen was 1 g./cu.m. Inhalation was for 4' 25", and the tracing, with points of blood sampling shown (B.S. 1, 2, 3), reproduced in fig. 2. Sample 4 was taken just before death, and is not shown on the tracing.

Time from zero.	A.P., mm.Hg.	Relative respiratory ventilation.	HCN content blood, μg./ml.	HCNS content of blood plasma, μg./ml.
Before . . .	210	30	0	3·9
35 sec. . . .	60	220	3·0	4·2
240 „ . . .	60	30	9·8	4·4
265 „ administration	ceased.			
375 „ . . .	20	0	6·2	5·0

Clearly, the concentration of HCN produced in the blood during the inhalation of the CNCl was of an order of magnitude such as to compare with that resulting from the inhalation of HCN itself. The concentration of HCN in the air inhaled was chemically equivalent to 75 per cent. of the CNCl used in the previous experiment: we may assume that half the concentration of HCN would have produced a maximal content of HCN of about the same order as was formed in the CNCl experiment, which would support our other estimates that about 30 per cent. of the CNCl is converted to HCN.

IV. Effect on the Central Nervous System.

In considering the effect of CNCl on the central nervous system, it must be borne in mind that most of the experiments here described have necessarily been performed on anæsthetised animals. Consequently the convulsions, and the vomiting, such prominent features when intact animals are used, are commonly in abeyance. Fig. 6, which is typical, shows that under these conditions general muscular movement is, on the whole, slight. Sometimes there are pre-mortal spasms when the anæsthesia is light, and a much reduced type of spasm may occasionally be seen in the spinal or decerebrate animal, from which the volatile anæsthetic used for the initial operation has been blown off, but these never have the tempestuous character of the convulsions seen in the intact unanæsthetised animal. It may be inferred from this that the convulsions are mainly of cortical origin, and that the remaining motor centres only play a subsidiary part in their production.

The initial effects of CNCl are often to produce facilitation or excitation of the bulbar and spinal centres, with resulting outbursts of activity. So, as with oxygen lack in general, there occurs a sympathetic outflow, with such symptoms as pupil dilatation, vasoconstriction, etc., also cardio-inhibition of central origin, as well as excitation of the respiratory centre by way of the outlying reflexogenic structures. Very soon the state of excitation gives way to one of paralysis, which probably spreads from the higher to the lower centres. Loss of consciousness comes on early, the conjunctival reflex disappears, the respiratory centre is depressed and probably the chemo-receptors fail, vaso-motor tone is reduced, and spinal reflexes are abolished. This latter happens quite early, as is illustrated by fig. 14 in which the knee-jerk was elicited by rhythmic taps on the patellar tendon with an electromagnetic hammer, as described by Schweitzer and Wright [1936]. The reflex is reduced before respiration fails, but returns abruptly (fig. 14) soon after the end of a small dose. Convulsions, when they occur, come on at a much later stage than that of the loss of the spinal reflexes, and it seems fair to assume that the latter is to be attributed to failure of synaptic conduction rather than to paralysis of the neurocytons themselves.

After a large or prolonged dose of CNCl such as leads to protracted gasping or failure of respiration from which resuscitation is only feasible with the aid of artificial respiration, recovery of the central nervous system is much slower and less perfect, and cyton damage is to be suspected. Hence, after apparent recovery from such a dose, the effects of further dosage may be modified. After such near-lethal exposures, particularly if they are repeated, there is always the possibility of permanent damage to the central nervous system, as, indeed, is the case in CO or HCN poisoning, or any other forms of anoxia. We have

only seen one such case with CNCl, and that was perhaps complicated by the fact that, to obviate discomfort, the animal (dog) was lightly narcotised with nembutal before exposure. This animal had received previous exposures to HCN 25–50 mg./cu.m. for 5 hours, and previous narcotisations with nembutal, also small doses of NaCN by mouth, without any after effects whatever. The exposure was to the low concentrations of 50 mg./cu.m. for 3 hours, followed by 100 mg./cu.m. for a further 3 hours; during exposure there were no convulsions, and no symptoms beyond rather deep and regular breathing after the first
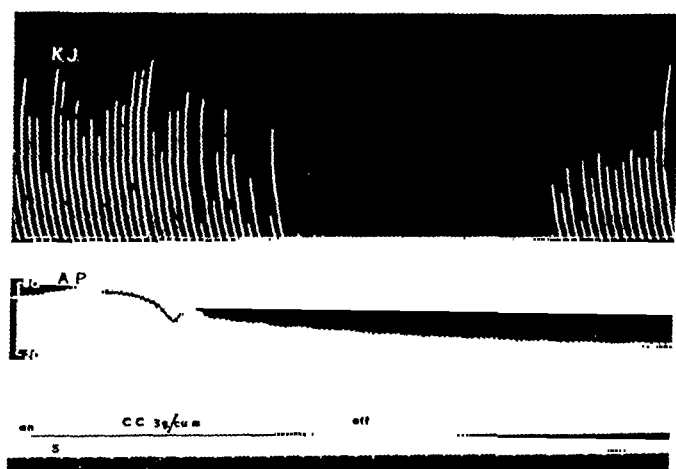

Fig. 14.—Decapitate cat (1 hour after decapitation). Knee jerk (K.J.) by taps at 26 p. min. Arterial blood pressure (A.P.). Inhalation of CNCl for 100 sec.

two hours. The dog, however, remained totally unconscious for 4 days, and only very slowly recovered smell, sight, hearing, and power of voluntary movement. Full restoration of voluntary movement and of hearing were apparently achieved after about 2 to 3 weeks, but even after 3 months vision was restricted, and the animal showed an altered disposition, being at times surly and unco-operative, with signs of mental degeneration.

We have seen two cases of hind-limb paralysis in dogs similarly treated with HCN. Oster, Toman, and Smith [1944] also report occasional blindness, loss of reactions, and behaviour-changes in cats repeatedly exposed to hypoxia.

V. EFFECT ON OXYGEN USAGE BY BODY.

From what has been said, it would be anticipated that CNCl would exert on the tissues an effect similar to that of HCN, and cause a reduction in the oxygen utilisation of the tissues, and so of the body as a whole. Experiment showed that this is so.

The arterio-venous oxygen difference was first examined. Samples of blood were simultaneously withdrawn from the venous and arterial sides, before and during administration of CNCl. In one experiment the jugular vein and carotid artery were the sites of sampling, and the degree of oxygen difference gave an idea of the oxygen utilisation of the head: in two other experiments blood was drawn from the right and left sides of the heart, so giving a measure of the general oxygen usage of the body as a whole. Even without analysis it was evident that during the administration of CNCl the oxygen uptake of the tissues was lowered, since the venous blood became brighter in colour, as in HCN poisoning.

The results are given below. The difference in oxygen content of the arterial and venous blood was determined by the Barcroft differential method, which was accurate enough for purposes of demonstration. The CNCl, 3 g./cu.m., was given by inhalation. For comparison a small dose of HCN was given in one case, by intravenous injection.

Site of blood sampling.	Time, min.	A–V difference. Vols. O_2/100 blood.
I. Ext. Jug.–carotid .	0	10·4
	15 (CNCl on)	..
	20	5·9
II. Right–left ventr. .	0	14·0
	2 (CNCl on)	..
	3·5	1·0
	4 (CNCl off)	..
	20	4·5
	27 (CNCl on)	..
	27·5	2·7
III. RV–LV . .	0	12·8
	4 (CNCl on)	..
	5 (CNCl off)	..
	5·5	3·2
	37	5·9
	41 (CNCl on)	..
	44 (CNCl off)	..
	44·5	0·6
	96	12·0
	103 (2 mg. HCN)	..
	104	3·0

The experiments show that the oxygen usage is not only depressed at the time of administration, but that the effect lasts for a considerable time afterwards.

Actual Oxygen Intake of Body.—The oxygen utilisation of the entire animal (rabbit) was determined, and graphically recorded by means of an improvised modification of the spirometer method of Krogh. The

CNCl was given in small doses (2 mg. at a time) by intravenous injection.
Fig. 15 shows the result of an experiment, and shows, even at a glance,
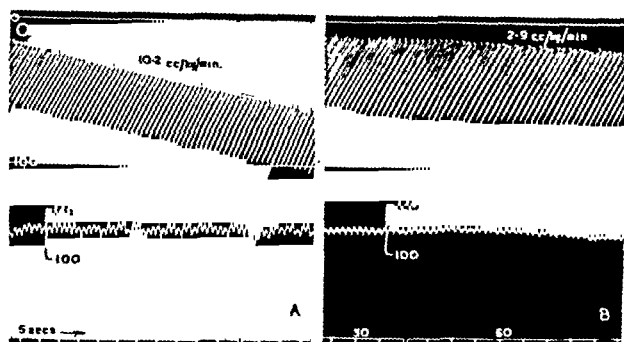

Fig. 15.—Rabbit. Nembutal. Oxygen usage by spirometer method. Figures
above spirometer record show oxygen usages in c.c./kg./min. A, normal period.
B, 25 sec. and onwards after second of two doses of 2 mg. each, of CNCl in 1 c.c.
saline, injected into external jugular vein.

that the oxygen uptake of the animal is greatly reduced after admini-
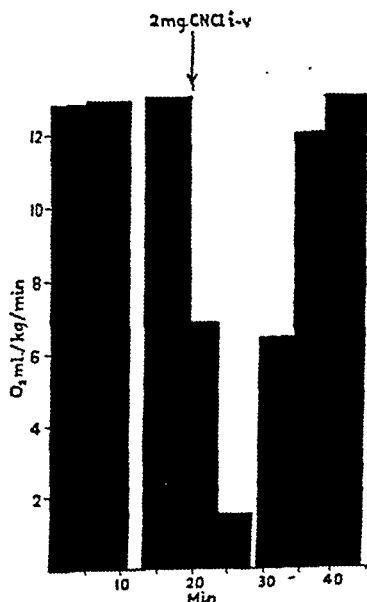stration of cyanogen chloride. The rate before administration was

Fig. 16.—Effect on oxygen consumption of rabbit. 2 mg. CNCl injected
intravenously at arrow.

steady, and about 10·2 ml./kg./min.; two successive doses of 2 mg.
CNCl were given with an interval of 5 minutes: the first dose caused
a drop to about 8 ml./kg./min., and the second to about 2·9 ml./kg./min.,

yet the arterial blood pressure showed little alteration, and the respiratory movements were not greatly increased. The results of another experiment, in which the effect of, and recovery from, a single dose of 2 mg. is well shown, are given in the graphs of fig. 16.

VI. Mode of Detoxication in the Body.

If, as we believe, a part of the cyanogen chloride introduced into the body is converted into HCN, its ultimate fate in the body should be the same as that of the latter substance. It is generally believed, and with some amount of direct evidence, that HCN is converted, at least in part, into thiocyanate in the body, and principally in the liver. Using the new and delicate method for estimation of cyanide and of thiocyanate (the latter only possible in the plasma), we have first obtained confirmation of this, by two independent methods. In the first, excised rabbit livers were perfused at 37° C., through the portal vein, with defibrinated blood, to which small amounts of cyanide were added from time to time; samples of the blood were taken at intervals for estimation of cyanide and of plasma thiocyanate. Some of the cyanide was lost by evaporation in the course of the circulation, so that figures for the extent of conversion cannot be given. The following outline protocol shows that conversion to thiocyanate did occur. The total blood volume was 170 ml.

Time, min.	Addition of HCN, (NaCN) μg.	HCN content (blood), μg./ml.	HCNS of Plasma, μg./ml.
0	100	0·6	1·5
5	slow addition calculated to give, in all, 30 μg./ml.	..	..
15	..	7·8	2·6
30	..	4·7	3·9
40	..	2·2	7·6

The second method depends on the fact that the saliva of the dog normally contains but little thiocyanate (it is usually stated to contain none). First it was shown in acute experiments that the saliva (obtained by chorda stimulation from the cannulated submandibular gland) contained as a rule less thiocyanate than 5 μg./ml., then, that after giving thiocyanate by mouth, or intravenously, so that the blood plasma contained, say, 20 mg./ml., the chorda saliva, sympathetic saliva, reflexly secreted saliva, pancreatic juice (by secretin), and urine all contained thiocyanate in large amount.

Next, a permanent salivary fistula (parotid) was established surgically under full nembutal anæsthesia in an 18-kg. bitch, so that a series of observations could be made, each extending over a period of days, if

.necessary. Saliva was collected by allowing the dog to eat meat, or by exhibiting meat to her.

Experiment 1.

Saliva by feeding contained HCNS = 3·5 μg./ml., at 4 p.m., 22.5.44.

At 5 p.m. gave 1 g. NaCNS in milk.

23.3.44.   ˙10.30 a.m.   Saliva = 63 μg./ml.   Blood 38 μg./ml. (5 p.m.).
24.5.44.   10 a.m.   Saliva = 22   ,,
25.5.44.   3 p.m.   Saliva = 16   ,,   Blood 12 μg./ml.
30.5.44   3 p.m.   Saliva = 4   ,,

Experiment 2.

15.6.44.   Normal saliva thiocyanate 1·8 μg./ml.
16.6.44 a.m.   ,,   ,,   ,,   3·2   ,,
17.6.44 p.m.   ,,   ,,   ,,   2·2   ,,
18.6.44.   Gave between 10 a.m. and 4 p.m. 15 doses, each of 10 mg. of NaCN, in 20 ml. milk. At 5 p.m. thiocyanate = 6 μg./ml. saliva.
19.6.44.   Saliva thiocyanate at 11 a.m.   = 16·5 μg./ml.
                                at 3·30 p.m. = 10·6 μg./ml.
20.6.44.   12 noon, saliva thiocyanate   = 8·7 μg./ml.
21.6.44.   11.30 a.m. saliva thiocyanate = 2·2 μg./ml.

Experiment 3.

Saliva thiocyanate = 4·3 μg./ml. at 10 a.m., 30.6.44.

30.6.44.   At 11 a.m. animal placed in 10 cu.m. chamber, and HCN conc. of mean 49 mg./cu.m. put up, till 11.40, when vomiting occurred, and respirations were deepened. The dog was removed for a spell, and replaced in concentration of 25 mg./cu.m. from 12.10 till 4.50, which was well tolerated.

30.6.44.   5 p.m.   Saliva thiocyanate = 16·5 μg./ml.
1.7.44.   5 p.m.   ,,   ,,   = 15·5   ,,
2.7.44.   10.30 a.m.   ,,   ,,   = 12·2   ,,
3.7.44.   ,,   ,,   ,,   ,,   = 7·0   ,,
6.7.44.   ,,   ,,   ,,   ,,   = 0·3   ,,

These three experiments show that thiocyanate given by mouth is excreted in the saliva (and other secretions), that clearance of the substance takes some days, and that HCN, given orally or by inhalation, leads to an increase in the excretion of thiocyanate in the saliva. Administration of CNCl was next proceeded with, but, in order to obviate suffering in our very co-operative collaborator, she was first given a light dose of nembutal, under which she slept well throughout the exposure.

31.7.44.   Saliva thiocyanate = trace less than 0·5 μg./ml.
1.8.44.   In the 10 cu.m. chamber, a conc. of 60 mg./cu.m. of CNCl was set up, and we, with another observer, went into it for some minutes to find whether it was unduly irritating,

which it was not held to be. At 11.05 the dog was brought in, and the conc. had now fallen to 41 mg./cu.m. The conc. was boosted up at intervals, to keep it at about 40–50 mg./cu.m., and, as this seemed to produce no effects, it was stepped up to 100 mg./cu.m. at 2.45 p.m. and kept at this level (81–105) until 5.30 p.m., when the dog was finally removed. The only symptom seen was deep breathing during the final 2 hours, but this was quite regular and there was no sign of convulsions.

2.8.44.   10.30 a.m. animal still unconscious, and had passed no urine. Saliva obtained by placing dilute acetic acid in mouth: thiocyanate = 34·5 μg./ml.

3.8.44.   10 a.m. still unconscious. Thiocyanate = 35·2 μg./ml.

4.8.44.   9.45 a.m. still unconscious. Thiocyanate = 29·2 μg./ml. Given water by stomach tube, and 2 doses coramine 1·7 ml., without effect.

5.8.44.   Unconscious: rigid: no reflexes obtainable. Given milk and Lemco by stomach tube. HCNS = 26·9 μg./ml.

11.8.44.   Periodic fits of tachypnoea and muscular rigidity, but slight improvement. HCNS = 18·9 μg./ml.

14.8.44.   Some improvement: can stand a little: has slight hearing, but still blind: refuses meat, but slowly laps milk.

21.8.44.   Walks and eats: has hearing, but still blind. Saliva thiocyanate = 21 μg./ml. Serum HCNS = 4·1 μg./ml.

30.8.44.   Vision returning. Walks and eats well.

20.10.44.   Vision still imperfect. Animal, formerly very friendly and demonstrative, is now surly and of senile appearance. Experiment discontinued.

Although the experiment succeeded in showing an increase in saliva thiocyanate following the exposure, its persistence is unexplained. For weeks after the experiment the saliva still contained an abnormally large quantity of thiocyanate, yet the diet was no different from that previously consumed. At first we thought that the kidneys were damaged, but it is difficult to suppose that their functions were so greatly impaired as the persistence would suggest, and, moreover, the plasma HCNS was not high later on.

VII. Experiments with Blood *in Vitro*.

Experiments were carried out *in vitro* in order to confirm the observations made *in vivo*, and, if possible, to discover something of the mechanism of the conversion. Here, we shall only deal with the first of these two goals: the second and more purely biochemical one will be the subject of a further paper.[1]

[1] By W. N. A. in preparation.

Pending the appearance of this communication, brief summary of the conclusions to date may be given. The conversion is a two-stage reaction: first the CNCl forms a combination with hæmoglobin: then, in presence of reduced glutathione HCN is liberated from this compound.

When HCN is added to blood, it remains unchanged, apart from some initial loss due perhaps to evaporation in mixing, during a considerable time. The following results are for defibrinated rabbit blood, 17° C:—

Time, min.	HCN, μg./ml. in			HCNS, μg./ml. in plasma.
	Blood.	Red cells.	Plasma.	
0	22	35	16·2	0·6
20	13·3	17·3	11·4	0·6
40	11·7	12·2	11·6	0·16
60	13·1	16·1	11·8	0·6
80	13·5	16·4	12·2	0·6

In contrast, when CNCl is added to blood at room temperature, it soon disappears, and some HCN replaces it. Thus, for defibrinated rabbit blood at 17° C.:—

Time, min.	CNCl, μg./ml. in blood.	HCN, μg./ml. in blood.	Per cent. conversion CNCl–HCN.
0	21·2	0	..
15	0	2·74	29·5
30	0	2·36	25·4

The reduction in HCN after 30 min. is possibly due to evaporation.

In a series of similar experiments, varying amounts of CNCl were added to defibrinated rabbit's blood (37° C.) with the following results:—

CNCl added, μg./ml.	Per cent. conversion.	CNCl added, μg./ml.	Per cent. conversion.
130	32	22	36
70	39	21	32
62	30	18	33
30	39	9·6	59

When serum was used instead of blood, however, the CNCl was again very rapidly destroyed, but now no HCN or HCNS was produced.

Evidence that HCN was produced by the action of blood on CNCl was provided by the observation that the substance in blood treated with CNCl not only gave the colour reaction with amines in pyridine

solution, after preliminary bromination, by which the estimations were made, but also gave the copper benzidine, Prussian blue, and thiocyanate reactions.   Blank tests without blood were, of course, negative.   The conversion of CNCl into cyanide evidently only occurs in presence of red cells, and the serum or plasma, if anything, competes with the red cells, and destroys the CNCl by conversion into something (at present unidentified) other than cyanide.   For this reason, the percentage conversion to cyanide may be greater when washed red cells suspended in saline are used than when whole blood is taken: the following figures, from a series of experiments with such suspensions, illustrates this:—

Experiments at Body Temperature (37° C.).

CNCl added, μg./ml.	Per cent. conversion.	μg./CNCl/ml.	Per cent. conversion.
126	25	20	61
65	45	20	59
33	68	9·7	73

The mechanism of the change need not be considered here, but that in whole blood it is extremely rapid is shown by the following experiment: For each test, 1 ml. defibrinated rabbit blood was warmed to 37° C. in a small centrifuge tube.   At zero time, 0·2 ml. of an aqueous CNCl solution, containing 11·5 μg./ml., also previously warmed to 37° C. was added from a syringe: the whole was rapidly mixed, kept at 37° C., and at a prearranged time the reaction was stopped by the addition of 5 per cent. trichloracetic acid.   Blanks were done with CNCl and trichloracetic acid.

Time, sec.	Per cent. conversion.
5	57·5
10	59·3
30	55·7
60	50·0
600	46·2

In from 5 to 10 secs., the maximal amount of conversion (greater than customary) had taken place.   It will have been noted that when the amount of CNCl added is relatively small, the degree of conversion is higher, as in the present experiment, though it is seldom less than 30 per cent.   What degree of conversion obtains for CNCl injected or inhaled into the blood-stream cannot be exactly stated.   Some of the data already given also suggest a figure of the order of 30 per cent. But toxicity figures, on the assumption that the toxicity is due solely and entirely to the HCN formed, would suggest a higher figure.   Thus,

the LD_{50} for rabbits, for HCN by intravenous injection is about 0·8 mg./kg., while trials with CNCl showed it to be of the order of 2·5 mg./kg. This would, on the assumptions given above, amount to a conversion of about 75 per cent. of the CNCl into HCN.

SUMMARY.

, 1. An analysis of the physiological effects of cyanogen chloride is given. These effects closely resemble those of hydrocyanic acid: in addition there is a local effect on the respiratory passages, resulting in broncho-constriction and ultimately in damage, when the vapour is inhaled.

2. Blood, or red corpuscles, rapidly converts cyanogen chloride into hydrocyanic acid. Serum also rapidly destroys cyanogen chloride, but without formation of hydrocyanic acid.

3. Conversion of CNCl into HCN by blood is not quantitative. *In vitro* the per cent. conversion is about 30: for isolated red cells it may be more, and it may be more *in vivo*.

4. When CNCl is inhaled, HCN can be immediately identified in the blood, and in amount comparable with that detectable when comparably toxic concentrations of HCN are breathed.

5. It is concluded that the lethal effects of CNCl are due to the formation of HCN, and that other actions are subsidiary.

6. Evidence is given that both HCN and CNCl are ultimately converted into HCNS in the body.

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THE RELATIONSHIP OF HEPARIN TO TRAUMA. By JOHN POLLARD and S. BRANDON STOKER. From the Department of Physiology, King's College, Newcastle-on-Tyne.

DURING a study of the blood clotting time (B.C.T.) it was found that, on our apparatus, in normal healthy persons values from 3 to 15 minutes occurred [Stoker, 1934]. In any one person B.C.T. is not constant. Changes, often periodic, are produced naturally which sometimes amount to three or four minutes. These periodic changes take place over several hours, and are best observed when specimens are taken from a person at 15- to 20-minute intervals. When specimens are taken at short intervals such as 1–3 minutes, we have found that changes of a different kind occur as shown in fig. 1. It will be seen that after a preliminary shortening of C.T. there is a general rise which lasts about seven minutes. Such results suggested that the trauma caused by taking the blood sample introduced changes in B.C.T. of a different order from the naturally occurring changes referred to above. A group of experiments in which two tests only were made, the second at varying intervals after the first, showed that such changes in C.T. are set in motion by the first prick and are of the type shown in fig. 1 whether the first prick is followed by one or by several further pricks soon afterwards. This investigation was undertaken to explore such changes in clotting time due to trauma. The causative agent of the preliminary shortening will be referred to as Discharge A, and that of the anticoagulant effect as Discharge B. Discharge B will be considered first.

Since it seemed that heparin, which has been the only known naturally occurring anticoagulant, might be involved, we have made a detailed study of its action *in vitro* in quantities capable of producing effects comparable with the observed differences in B.C.T. We have also investigated the action of the basic anticoagulant Toluidin Blue and the acidic anticoagulant Chlorazol Fast Pink. The choice of solvent for these substances is a matter of importance. The changes in C.T. to be investigated seldom exceed four minutes, and addition of saline may itself cause lengthening, while distilled water may cause a slight shortening. We found it advantageous to work in distilled water solution, and the tests in this work, except where otherwise stated, have been made with 85 volumes of shed blood and 15 volumes of aqueous addition. Our

tests have been made on capillary blood run directly from the skin puncture into the cannulæ, except for a few tests on animals in which venous blood was used.

In view of the smallness of the changes to be investigated, it was necessary to take all possible steps to ensure accuracy. We have used the Stoker Automatic Multiple Coagulometer [1945] which gives readings at half-minute intervals, and closely adhered to the prescribed technique. We maintained a room temperature of approximately 70° F., a very high degree of cleanliness of apparatus, and constant dimensions and quality
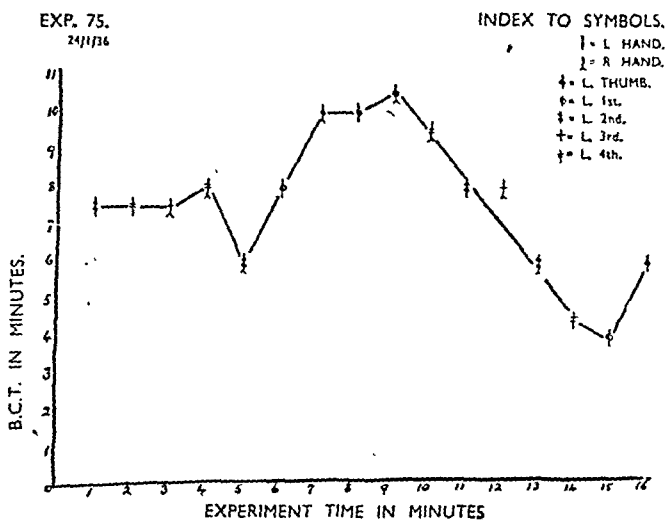

FIG. 1.—Clotting times of capillary blood unmixed with any diluent.

of glass cannulæ, since we find that different qualities of glass give different values for C.T.

Two cannulæ are prepared. Into one is introduced a solution of the anticoagulant, and into the other distilled water or normal saline according to the solvent used for the test substance. Blood obtained from one flow is run into each and the contents of each cannula are then carefully mixed. The specimen with aqueous or saline addition only is used as a control, and the difference between the clotting times gives the effect produced by the added substance. Our ultimate objective being the investigation of the effect of trauma, we have avoided, as far as possible, using for these tests the blood of persons affected by even slight injuries.

Arrangement of Results.

When equal amounts of an anticoagulant are added to specimens of blood the control samples of which show differing C.T., the effect of the anticoagulant is not always the same but varies according to the C.T. of

the control. Figs. 3 and 4 show that by arranging results in order according to the C.T. of the controls a regular sequence of effect is obtained. Results varying in this manner are given by the basic anticoagulants Tol. Blue and Methylene Blue, and by the acidic anticoagulants heparin, Chlorazol F. Pink, and Chlorazol Sky Blue.

Toluidin Blue.—The work falls into three parts:

(*a*) With quantities exceeding 35 parts per million parts of blood (parts per mill.), the well-known anticoagulant effect can be seen. This action is not discussed here. (By 1 part per mill. is meant 1 mg. per 100 ml. blood.)
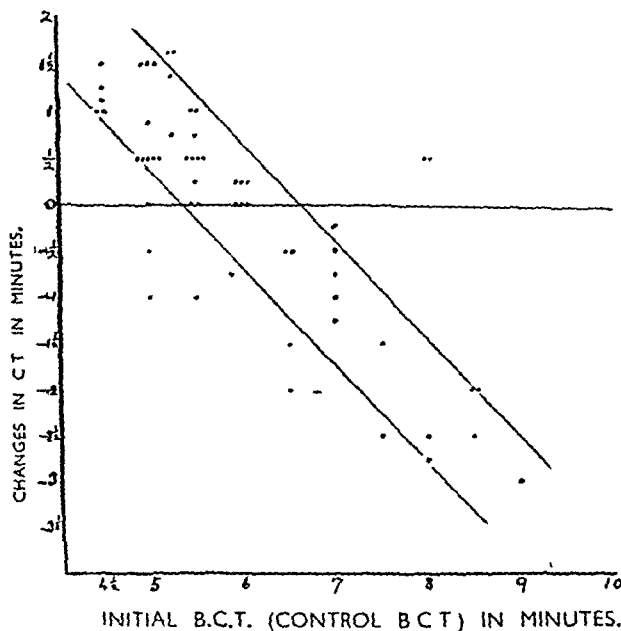

FIG. 2.—Effect of Toluidin Blue, in strengths varying from 12 to 25 parts per million in the blood, on blood samples of various clotting times.

(*b*) With amounts of Tol. Blue between 12–25 parts per mill. the effect is to bring all C.Ts. to around 6 minutes whatever the *control time* may be. Small differences occur, but the physiological significance of these cannot be determined with validity by present methods. In fig. 2 each dot represents the result of one test, and 85 per cent. of the dots lie in a straight diagonal band.

(*c*) In quantities of 1, 2, and 3 parts per mill. Tol. Blue has an action resembling but more powerful than that described under (*b*). The results are given in Table I. Minute quantities of adrenalin and the trauma discharges are sufficient completely to alter the nature of the effect. Consequently tests can be successfully made only with blood

TABLE I.—THE ACTION OF TOLUIDIN BLUE SALINE SOLUTION ON BLOOD OF VARIOUS C.Ts. SHOWN AS CHANGE, IN MINUTES, FROM CONTROL.

B.C.T. of specimens in minutes.	7.	7½.	8½.	9½.	
Amount of Toluidin ⎧ 1	+4¼	+3	0	−3½	Change in mins.
Blue in parts per ⎨ 2	+2½	+1¾	−½	−3	from control.
mill. of blood. ⎩ 3	+1½	+2	−½	−4	

from persons as free as possible from tension of mind and body and free from injuries.

3 parts per mill. has a much greater action than that of 12–25 parts per mill., but the greatest action is given by 1 part per mill. This is the
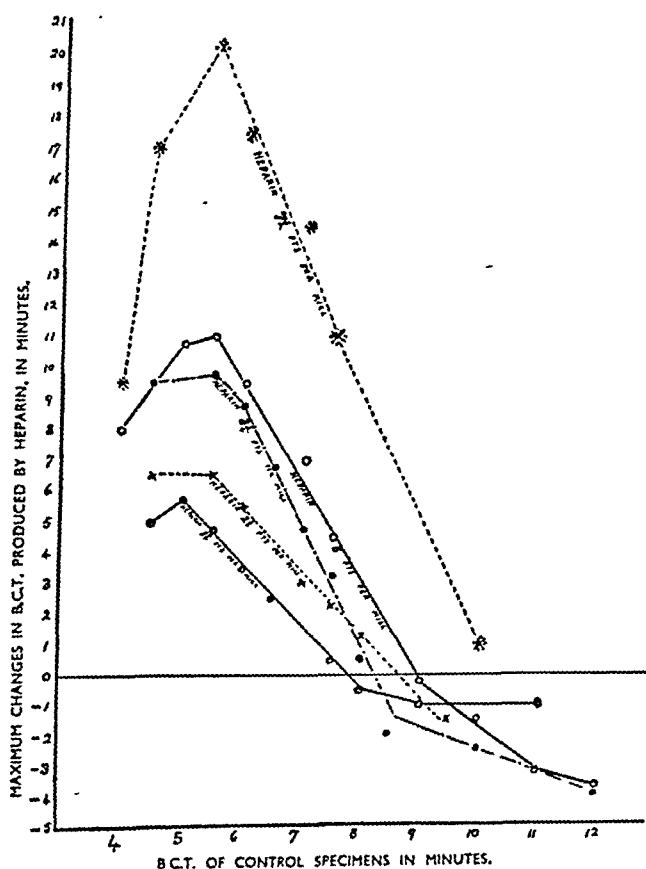
CHART OF MAXIMUM HEPARIN EFFECTS ON B C.T

FIG. 3.—Maximum effects of heparin, in strengths of 1¼, 2½, 3¾, 5, and 7½ parts per million of blood, on blood of various clotting times.

concentration displaying the maximum "irregular sequence" effect to which we make further reference when considering the action of heparin.

The behaviour of Tol. Blue suggests that higher clotting times are connected with a higher concentration of acidic substances in the blood. This is supported by the work of Lowndes Yates [1928], who found that long B.C.Ts. could be shortened by the ingestion of sodium bicarbonate until the urine became alkaline.

Heparin.—Using the same method we have investigated the action of heparin in strengths of 7½ parts per mill. and less. (The heparin was obtained from B.D.H. and was 111 Toronto units per mg.) Maximum results on normal people are shown in fig. 3. The effects on C.T. of
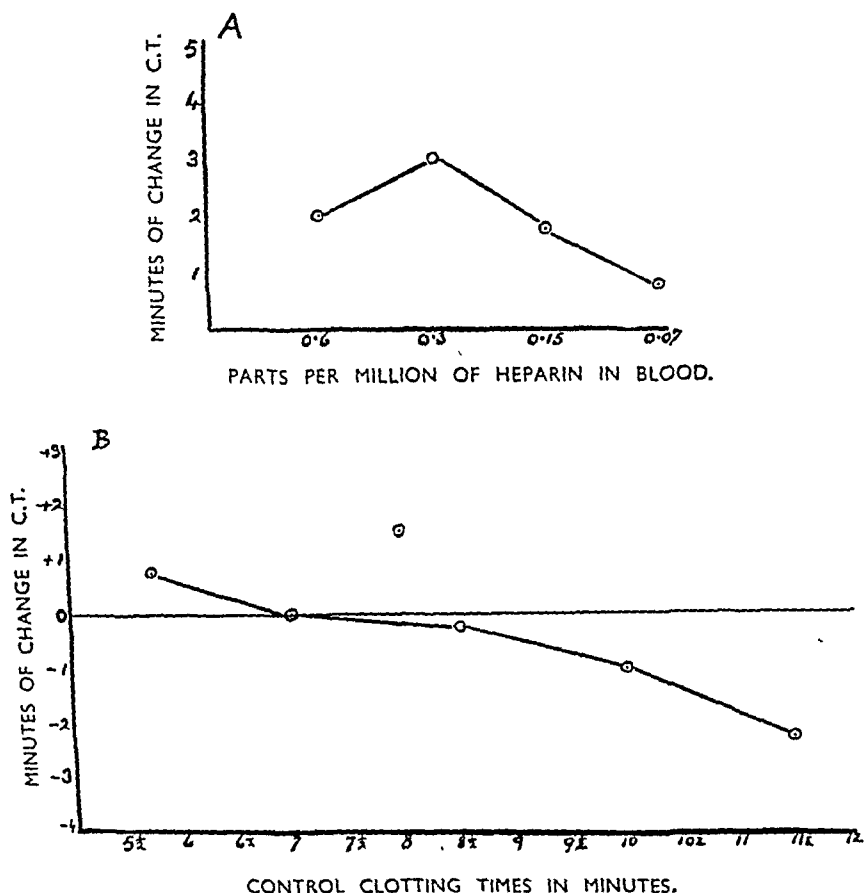

Fig. 4.—Part A. The action on blood, of 5½ minutes C.T., of heparin in strengths from 0·6 to 0·07 parts per million of blood.

Part B. The action on blood, of various clotting times, of heparin in the one strength of 0·07 part per million of blood.

In these tests saline was used as the heparin solvent and for the control samples.

TABLE I.—THE ACTION OF TOLUIDIN BLUE SALINE SOLUTION ON BLOOD·OF VARIOUS C.Ts. SHOWN AS CHANGE, IN MINUTES, FROM CONTROL.

B.C.T. of specimens in minutes.	7.	7½.	8½.	9½.	
Amount of Toluidin ⎰1 Blue in parts per ⎱2 mill. of blood. ⎰3	+4¼ +2½ +1½	+3 +1¼ +2	0 −½ −½	−3½ −3 −4	Change in mins. from control.

from persons as free as possible from tension of mind and body and free from injuries.

3 parts per mill. has a much greater action than that of 12–25 parts per mill., but the greatest action is given by 1 part per mill.   This is the
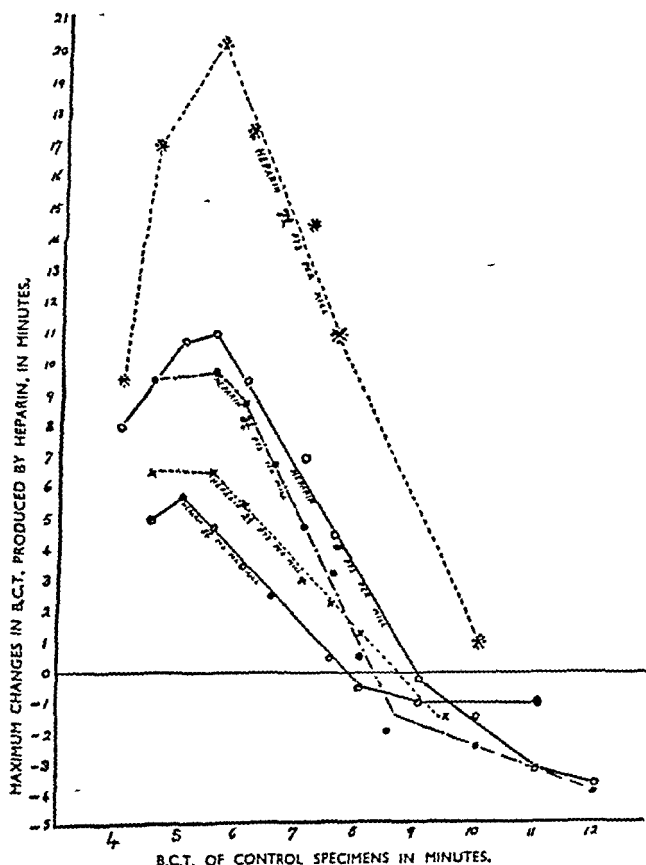

CHART OF MAXIMUM HEPARIN EFFECTS ON B.C.T.

FIG. 3.—Maximum effects of heparin, in strengths of 1¼, 2¼, 3¼, 5, and 7¼ parts per million of blood, on blood of various clotting times.

if heparin is added to blood *naturally* clotting at 8 minutes or over (always omitting blood affected by trauma) then the result is a shortening
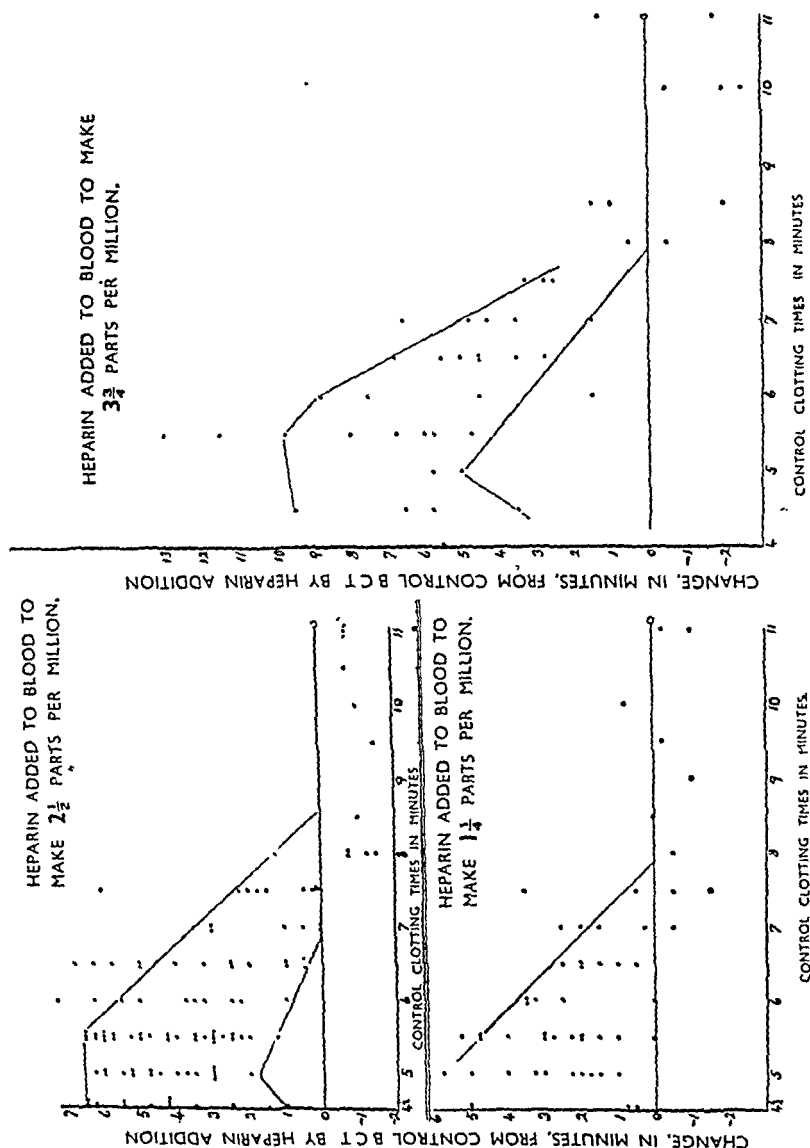

Fig. 6.—Scatter in the results when adding heparin to blood of various clotting times. These charts show the effects of heparin in strengths of 1¼, 2½, and 3¾ parts per million of blood. Each dot represents one test and shows the difference between the control and the specimen to which heparin has been added.

of C.T. This shows that the variations in C.T. found in trauma-free people are not caused by varying amounts of heparin but by some other factor.

In fig. 6 are given in detail the results on which fig. 3 is based. Each

different quantities of heparin are not in direct proportion to the amounts used. They are considered in more detail below.

Fischer [1931] using fluids other than fresh blood has shown that very small amounts of heparin may exert a more powerful effect than somewhat larger quantities. Heparin tests in quantities of 0·6, 0·3, 0·15, and 0·07 parts per mill. show that the "irregular sequence" effect is at a maximum around 0·3 parts per mill. *Maximum* effects are shown in fig. 4 (1 part of heparin combines with 4·1 parts Tol. Blue
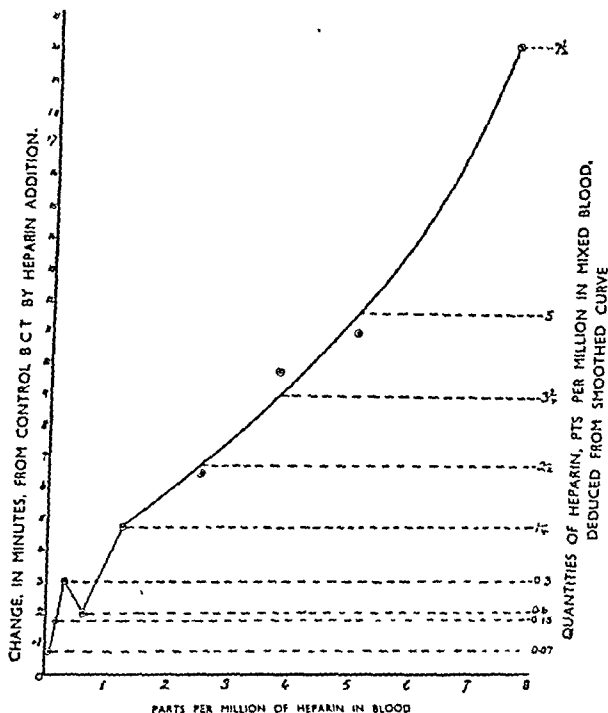

FIG. 5.—The action on blood, of 5½ minutes C.T., of heparin in strengths from 7·5 to 0·07 parts per million of blood.

[Jaques, 1943]). The quantities showing the "irregular sequence" effect are thus approximately chemically equivalent.

The action on blood of 5½ minutes C.T., of heparin in quantities from 7½ parts per mill. downwards is shown in fig. 5.

It will be seen that the action of heparin and of Tol. Blue is similar in that both substances cause a lengthening of short clotting times and a shortening of lengthy ones, but that there is a marked difference in the detailed shape of their activity curves.

As shown in fig. 3, blood of any lower C.T. may be made to clot at 8 minutes or over by the addition of a suitable amount of heparin. Addition of a further amount of heparin raises the C.T. still *higher, but*

blood on which it is acting, and the scatter is similar to that given by heparin.

In mixtures of heparin and Chlorazol F. Pink, Chlorazol F. Pink augments the action of heparin.

The Trauma Discharges.

We have used a prick for the dual purpose of withdrawing a specimen of blood and also for inflicting the trauma. The effect of the trauma has been measured by comparing the C.T. of the control sample of blood thus obtained with another sample taken subsequently. During the interval changes may have occurred due to causes other than trauma, but we have found such changes to have generally a negligible effect. The first prick dominates the chain of changes.

The first specimen, *i.e.* the control, may be taken from any convenient part of the body, usually a finger or toe. The second specimen may be obtained from any part of the body but should not be taken from the same digit as the control. To do so would introduce difficulties the nature of which will appear later. The changes observed, though differing in amount, are of the same type whether the blood specimens are taken from different fingers of the same hand, from the two hands or from a hand and foot, and are therefore changes in the circulating blood. We find that the nature of the effect is dependent on the C.T. of the control specimen. The type of change shown in fig. 1 is constantly found where the control C.T. is below 7 minutes, but where the control C.T. exceeds 8 minutes only a fall in C.T. usually results. In an experiment such as shown in fig. 1 the course of the rise in C.T. may be altered by reason of there being a number of pricks, but the changes occurring are actually initiated by the first prick of the series.

The lowest readings, found in 55 experiments made similarly to that represented in fig. 1, are shown in fig. 7. These correspond to Discharge A and are usually recorded within 4 minutes of the start of the experiment. The highest readings, in the same 55 experiments, are shown in fig. 8 and are usually recorded within 8 minutes of the start of the experiment. All the values shown in both charts were recorded within 10 minutes of making the first prick.

It will be seen that, in fig. 7, Discharge A gives an almost straight line of maximum effect which corresponds to the reduction of C.T. to around 5 minutes from all higher values. One test was obtained on blood of $4\frac{1}{2}$ minutes control time and one on blood of 4 minutes control time and in neither was a shortening of C.T. recorded (fig. 7, B). The inference is that Discharge A, on these qualities of blood, causes an increase, not a decrease in C.T., and hence its effect merges into that of Discharge B. Further discussion of Discharge A is given later.

The principal feature of the Discharge B graph is that there is a

dot represents one test. The most prominent feature of the charts is the scatter. In each case this is wide and yet compact where the action of heparin causes a lengthening of C.T., while where the C.T. is shortened the band of scatter is much narrower. (The number of tests shown here is much smaller because of the relative scarcity of blood of long C.T.) This suggests that the scatter has a definite significance.

Using blood of 5-minute control time as a standard of comparison, since this is the blood on which most numerous readings are available, and on which heparin exerts its maximum effect, the particulars in Table II are obtained.

TABLE II.

TAKEN FROM FIG. 6.	TAKEN FROM FIG. 5. Amount of heparin required to give these effects on C.Ts. of 5½ minutes.
With 2⅓ parts of heparin (fig. 6)— Maximum rise in C.T., 7 min. Lower limit of scatter, 1¼ min.	2·5 parts per mill. 0·1 part per mill. approx.
Approx. 2·4 parts per mill. of heparin would thus be required to raise the C.T. from the lower to the higher limit of scatter.	

If comparisons are made on the other parts of the charts similar results are obtained. In fig. 6, with 1¼ parts per mill. of heparin, the area of scatter extends down to the line of zero activity, and corresponds with the zone free from scatter with 3¾ parts per mill.

These findings lead us to the view that the scatter is caused by the occurrence in the blood, in varying quantities, of a substance or substances which neutralise the effect of heparin on C.T.

The presence of the anti-heparin bodies throws some light on the results shown on each chart around 7- to 8-minute controls. Here some tests show an increase, others a decrease. The lack of definition in the point of zero activity is shown to arise from wide variations in the amount of heparin which is actually affecting the C.T. With 0·15 part per mill. of heparin the point of zero activity is with blood of 6½ minutes C.T., while with 2½ parts per mill. it is above 8 minutes C.T.

The fact that there is no comparable scatter effect with Tol. Blue suggests that the anti-heparin substance or substances are of a chemically basic type (*cf.* fig. 2).

Action of Chlorazol F. Pink.—Over 40 tests of the action of this acidic anticoagulant show that when used in quantities of 60–150 parts per mill. its effect is comparable with, though not exactly parallel with, that of heparin. It lengthens or shortens C.T. according to the C.T. of the

marked increase in C.T. where the control is $7\frac{1}{2}$ minutes or less, but with controls of 9 minutes or over there is a definite shortening of effect.

On comparing fig. 8 with fig. 6, which gives the effect of $1\frac{1}{4}$ parts per mill. of heparin, points of similarity may be observed as follows:—

1. The general shape of the two curves is the same.
2. Using the $5\frac{1}{2}$-minute control column of readings for comparison, as was done with different quantities of heparin, we find the heparin readings show a maximum of $5\frac{1}{4}$ minutes, while the Discharge B readings rise to 5 minutes.
3. When considering the action of differing quantities of heparin, it was observed that the scatter results indicated the presence of anti-heparin factors in the blood. The depth and distribution of the scatter is the same in the trauma chart, fig. 8, and the chart showing the effect of $1\frac{1}{4}$ parts per mill. of heparin, fig. 6.

When discussing the effect of heparin we have mentioned the difficulty of determining the exact point of the zero activity of any given quantity of heparin owing to the effects of the presence of the anti-heparin substances. The same blurred effect is seen on the chart of the Discharge B activity; in the $8\frac{1}{2}$-minute control column the readings are distributed between $-1\frac{1}{2}$ minutes and $+4$ minutes. There is thus a very marked similarity between the two graphs.

The Nature of Discharge B.—It is well known that heparin in the blood will react with Tol. Blue. When the amount present is large an estimate of the quantity can be made by this means. When the amount is small the C.T. no longer varies directly in proportion to the neutralisation of the heparin. This is because of the complex nature of the action of Tol. Blue on the blood. But although its amount is difficult to estimate by this method its presence causes a noticeable effect. We have applied this test to blood after trauma.

Blood from a first prick is tested with Tol. Blue in three different dilutions, while to another portion of the same specimen only water is added. Blood from a later prick is treated similarly. The changes produced by the Tol. Blue on the blood from the first prick are compared with those produced from the second prick. The difference between the two sets is due to a change in the quality of the blood between the two pricks.

In Experiment 426, Table III, the rise of 3 minutes in C.T. from the first to the second prick suggests a relatively large inflow of an anticoagulant. Reference to the Tol. Blue charts suggests that the effects shown by the Tol. Blue are those of 1, 4, and 8 parts per mill. This means that the anticoagulant has combined with 10 to 11 parts of the dye, and is in harmony with the view that it is a heparin-like substance. Experiments are only rarely obtained in which the natural inflow of the
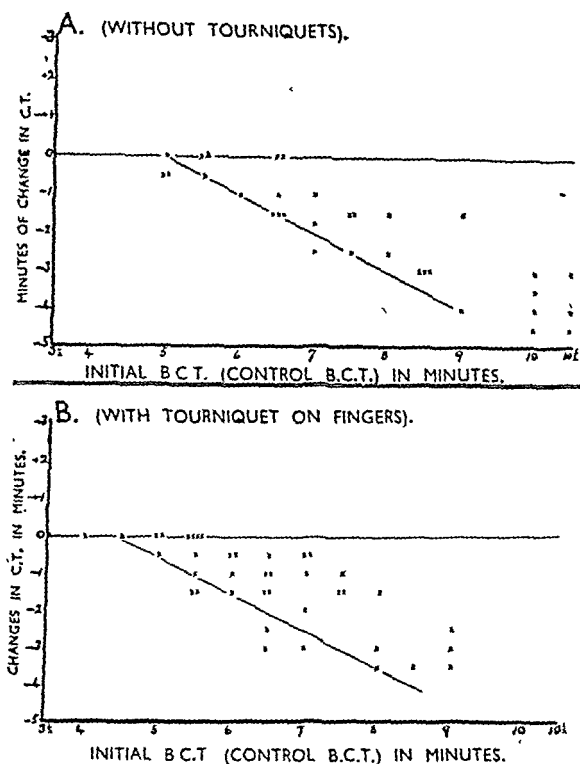
FIG. 7.—Showing the scatter and maximum effects of Discharge A when (A) the trauma is a simple prick to the finger and (B) when the prick trauma is augmented by the application of a tourniquet. Each cross represents one test. No test is shown on bloods with C.T. below 4½ minutes, as the effect of Discharge A is to lengthen the C.T. of blood clotting in less than 5 minutes.
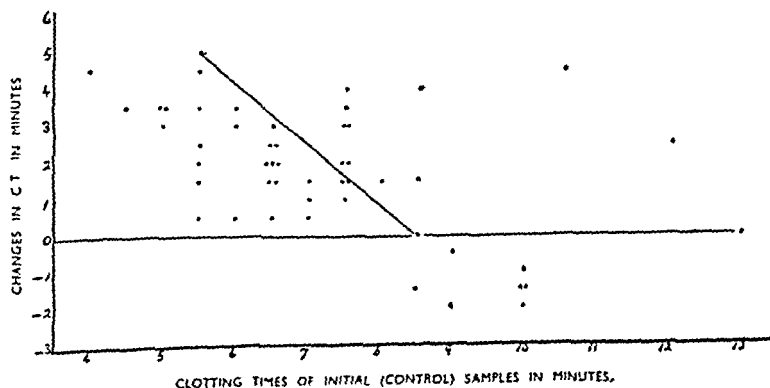

FIG. 8.—The scatter and maximum effects of Discharge B plotted similarly to the Discharge A and the heparin scatter charts.

that blood of 8 minutes C.T., or higher, may have that figure as its true basic clotting time, or that it could be attained by the addition of heparin to blood of lower basic clotting time.   If to blood of the first type there be added 1¼ to 2½ parts per mill. of heparin there would be a fall in C.T., while if added to blood of the second type the C.T. will be lengthened.   In our experiments, where the effect of trauma had raised the C.T. to over 8 minutes, we found that the addition of heparin raised the C.T. still further.

We therefore conclude that Discharge B is heparin or a heparin-like substance.

The Duration of Discharge B.—We have so far only considered the course of events during a period not exceeding 10 minutes from the
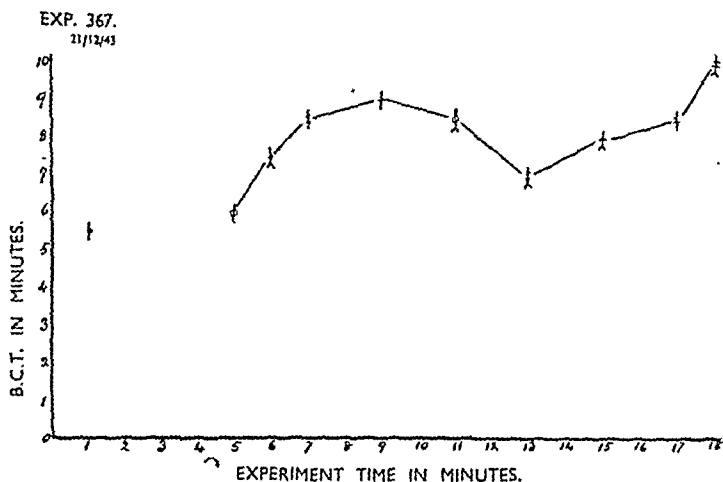

Fig. 9.—The details of this experiment are similar to those of fig. 1, but this experiment is over a longer period and shows two rises in C.T.

making of the first prick.   Referring again to fig. 1, it will be seen that after reaching a peak at the ninth minute the C.T. falls, reaching its lowest point at the fifteenth minute.   The last test given, at the sixteenth minute, shows it rising again.   In fig. 9 is given the result of a test in which, at the eighteenth minute, the C.T. has risen to a peak for the second time.   We find this to be the usual type of effect, though these two examples have been specially selected because they show the effect of the two discharges in a particularly clear manner.

It thus appears that Discharge B does not enter the blood continuously throughout the period of the infliction of trauma.   It comes, instead, in a series of intermittent flows, between each of which the C.T. decreases.   We have tried to ascertain the length of time elapsing before the effects of a prick cease to be discernible, and for this purpose have made use of added heparin.   As long as Discharge B is present the amount of free anti-heparin substances should be lower than before

TABLE III.

Expt. No.	B.C.T. of control.	Amount of Tol. Blue in parts per mill. of blood.		
		12.	15.	18¾.
426	2nd prick, 8 min. 1st ,, 5 ,, Difference 3 ,,	*B.C.T. of treated specimens.* 10¾ min. 6½ ,,	 8 min. 7 ,,	 5¼ min. 6½ ,,
430	2nd prick, 5 min. 1st ,, 5 ,, Difference 0 ,,	5½ min. 5½ ,,	4½ min. 4¼ ,,	4¼ min. 5 ,,
		1.	2.	3.
570	2nd prick, 8½ min. 1st ,, 7½ ,, Difference 1 ,,	*B.C.T. of treated specimens.* 7¼ min. 10½ ,,	 7¼ min. 9¼ ,,	 7¾ min. 9½ ,,

anticoagulant so nearly matches the amount of Tol. Blue used in the example, but we have many results which are in close agreement with it.

In Experiment 430, Table III, there is no difference between the two controls, suggesting that there is no trauma discharge. (This subject was tested on a number of occasions and regularly gave the same result.) The Tol. Blue action in both series gave the same results, confirming this view. In Experiment 570, Table III, the difference of one minute between the controls corresponds to that which would be given by about 2 parts per mill. of heparin. Examining the findings from the first prick one sees the results of the action of the Tol. Blue to be in accordance with expectations as shown by Table I. If 2 parts of heparin were acting with 1, 2, and 3 parts of Tol. Blue the whole of the dye would be neutralised, leaving an excess of heparin. The C.T. observed is in accordance with this view.

We have tested the effect of Chlorazol F. Pink on the trauma Discharge B. We have made 8 tests, and in each case the C.T. was altered in the same way as we found was produced by mixtures of heparin and Chlorazol F. Pink.

The Action of Added Heparin.—We have found, as indicated earlier

heparin per kilo of ox blood. This is equal to 0·6 part per mill. of heparin (111 Toronto units per mg.). This quantity is very much smaller than is contained in any of the organs except the thymus, and is in close agreement with the quantity of Discharge B expected. We feel that this finding gives additional support to the view that · Discharge B is of a heparin nature.

When heparin enters the blood much of it will be neutralised by Discharge A and by the anti-heparin bodies normally present. According to the Mass Law the amount of heparin entering into combination will vary according to the relative proportions of the heparin present and the substances with which it combines. It follows that the differences in C.T. are not a guide to the amount of heparin present unless the amount of anti-heparin substances is known. In attempting to estimate the amount of heparin present as Discharge B we have partially overcome this difficulty as follows: $1\frac{1}{4}$ parts per mill. of heparin are added to blood from a first prick and also to blood obtained some minutes later, the C.T. of each sample being recorded and also that of further parts of the same specimens without the addition of heparin. The four specimens are: (a) The blood from the first prick without heparin serves as control. (b) The first prick blood plus heparin gives a measure of the amount of anti-heparin substances present. (c) The second prick sample without heparin gives the ordinary trauma rise in C.T., but is not needed in the calculation. (d) The second prick sample plus heparin gives the combined effects of the Discharge B and the added heparin less the amount used in neutralising Discharge A and the anti-heparin substances. From our file particulars are selected of a test showing as nearly as possible the same control as (a), the same effect on addition of $1\frac{1}{4}$ parts per mill. of heparin as specimen (b), and in addition the effects of $2\frac{1}{2}$ and $3\frac{3}{4}$ parts per mill. of heparin. On comparing the C.T. of specimen (d) with the effects of $2\frac{1}{2}$ and $3\frac{3}{4}$ parts per mill. of heparin, the amount of heparin present as Discharge B can be estimated, but the value thus obtained will always be slightly low since no allowance has been made for the heparin neutralised by Discharge A. Later we give reason for thinking that the amount neutralised by Discharge A is small. Results obtained are shown in Table V.

In Expt. 509, Table V, the effect on C.T. of $1\frac{1}{4}$ parts per mill. of heparin was only $+1\frac{1}{4}$ minutes, whereas in the test under discussion the effect was $1\frac{3}{4}$ minutes. Hence the blood in Expt. 509 contained slightly more anti-heparin substances and a valuation based solely on it would be somewhat too high. It gives the amount of heparin necessary to produce a rise of $4\frac{1}{4}$ minutes as about $3\frac{1}{4}$ parts per mill., and so the amount present as Discharge B as 2 parts per mill. Using Expt. 514 similarly corrections have to be made because (a) the control is $5\frac{1}{2}$ minutes instead of 5 minutes, and (b) the addition of $1\frac{1}{4}$ parts per mill. of heparin lengthens C.T. by 2 minutes instead of $1\frac{3}{4}$ minutes as in the

it entered the blood, and consequently the effect of added heparin should be greater. The effects of such additions of heparin are given in Table IV. In each of the tests shown heparin was added to the

TABLE IV.

Expt. No.	Lapse of time in minutes.											
	0.	5.	10.	15.	20.	25.	30.	35.	40.	45.	50.	55.
439	100	190			100		89					
406	100		236	124		96						
438	100		126		28				106			
459	0		12									
466	0		24									
487	100			111								
490	100				293							
488	100										150	
492	100			85			89					90

Variations in heparin activity (calculated as a percentage of that shown by the first prick) with lapse of time.

blood from a series of pricks. The effect produced on each specimen of the series was calculated as a percentage of the effect produced on the first specimen of the series, except in Experiments 459 and 466 in which the first reading was nil; in these cases the second test is given as a percentage of the maximum activity.

The Discharge B effect is at a maximum after an interval of about 8 minutes between pricks and thereafter it diminishes. It has usually died away by the sixteenth minute, but in two exceptional cases it still shows, at a considerable level, after 20 minutes and 50 minutes respectively. Experiments 439, 438, and 492 show that where the effect of trauma has died away the effect of the added heparin reverts to the original level.

The Amount of Heparin as Discharge B.—When a large quantity of blood has been taken from an animal for an assay it may be assumed that the animal received appreciable injury. Discharge B should thus be present in the later stages of the flow, and become mixed in the specimen as a whole.

We have not fully investigated the amount of Discharge B following major trauma, but the results of a number of experiments on animals (not described here) lead us to the view that it may not differ greatly from that shown following a skin puncture. The amount in a large sample of animal blood should thus be small, probably less than 2 to 2½ parts per mill. which we calculate below to be present in blood at the time of maximum flow after the initial prick. Charles and Scott [1933] in an assay of the heparin content of various organs, found 66 units of

TABLE VI.

Expt. No.	Time of prick.	C.T. of specimens in minutes.		Change in C.T. in minutes.
439	Zero 4 min.	A 6 C 6½	B 8½ D 10½	From A to B 2½ From A to D 4½

Comparable test:—

Expt. No.	Control C.T. in minutes.	Minutes of change produced by heparin in quantities shown as parts per mill.		
		1¼ parts.	2½ parts.	3¾ parts.
509	5½	+2¼ ·	+5¼	+8½

made on blood clotting originally in 5 minutes, but whereas the first rose to 5½ minutes after trauma, the second rose to 8 minutes. In spite of this the different methods used point to approximately the same figure for the total amount of heparin present in each case. Expt. 439, Table VI, shows more than 1¼ parts per mill. after an interval of only 4 minutes.

The observed changes in C.T. provide a guide to the amount of free heparin present. In Expt. 426, Table III, the observed increase in C.T. is from 5 to 8 minutes, corresponding to the effect of between 0·3 and 0·6 parts per mill. of heparin. In Expt. 406, Table V, the change is from 5 to 5½ minutes, corresponding to the effect of 0·1 part per mill. of heparin. In Expt. 439, Table VI, the change is from 6 to 6½ minutes, corresponding to the effect of 0·1 part per mill. of heparin. Examination of fig. 8 shows that only rarely does the amount of free heparin exceed 0·8 part and that usually it lies between 0·1 and 0·8 parts per mill. In Expts. 406 and 439, Tables V and VI, the free heparin is approximately 5 per cent., and in Expt. 426, Table III, between 5 per cent. and 25 per cent. of the total present. These amounts are so small that they suggest the excess which would be required to prevent dissociation of a heparin compound. It would appear that the main function of the Discharge B is not to affect the clotting time so much as to combine with some other substance or substances.

Mechanism of Discharge B.—When the circulation to a finger or toe was occluded by a tourniquet (elastic band or pneumatic cuff) and then trauma produced, it was found (57 experiments) that the rise in C.T. is greater on the side of the body remote from the injury and least near the

TABLE V.

Expt. No.	Time of prick.	C.T. of specimens in minutes.		Change in C.T. in minutes.
406	Zero 8 min.	A (Control) 5 C (Effect of 5½ trauma)	B (+heparin) 6¾ D (Trauma effect 9¼ + heparin)	From A to B 1¾ From A to D 4¼

From the files two comparable tests are available as follows:—

Expt. No.	Control C.T. in minutes.	Minutes of change produced by heparin in quantities shown as parts per mill.		
		1¼ parts.	2½ parts.	3¾ parts.
509	5	+1⅓	+3	+5
514	5½	+2	+3¼	+6¾

specimen under test. The corrections are thus in opposite directions, approximately cancel out, and we arrive at the figure of 1¼ parts per mill. as the amount of heparin present as Discharge B. The two amounts given are thus in good agreement but, as already stated, no allowance has been made for heparin neutralised by Discharge A. Other calculations made by the same method give results varying from 1¾ to 3 parts per mill. of heparin.

The same method applied to blood specimens obtained at an interval of only 4 minutes gave results shown in Table VI.

We have also endeavoured to ascertain the amount of heparin by means of Tol. Blue. The heparin–Tol. Blue compound is known to be of low solubility, hence the addition of Tol. Blue to blood containing free and combined heparin will lead to the formation of heparin–Tol. Blue complex in preference to any other more soluble heparin compound. This will be followed by the dissociation of the anti-heparin–heparin compound, and freed heparin will combine with further Tol. Blue. Ultimately the greater part, possibly the whole, of the heparin will combine with the Tol. Blue. The use of Tol. Blue should thus make possible an estimate of the whole of the heparin present, whether free or combined, provided an indication of the end of the reaction can be obtained. This is supplied when the combination of Tol. Blue proceeds to the point at which the "irregular sequence" effect can be recognised. Expt. 426, Table III, shows this effect, and the data given correspond to the presence in the blood of approximately 2½ parts per mill. of free and combined heparin. Expts. 406 and 426, Tables V and III, were both

The effect of adrenalin hydrochloride on human blood *in vitro* was examined by adding it in quantities to give final concentrations of 1 in 6 millions down to 1 in 40 millions. It was found that all blood, whatever its initial C.T., and whatever the quantity of adrenalin within the limits quoted, was made to clot in 5 minutes. In this respect the action of adrenalin is similar to that of Tol. Blue and also to Discharge A.

Adrenalin hydrochloride (1 : 1000) has been injected subcutaneously, in doses ranging from $\frac{1}{2}$ to 7 minims into nine apparently normal human subjects. The subcutaneous injection of adrenalin is painful and hence will cause the release of considerable Discharge A. Any effect in other directions will thus have superimposed upon it the effect of this Discharge A. The changes in C.T. were consistent with the presence of adrenalin or with Discharge A in amounts larger than found in our other experiments. In none of these cases did the C.T. suggest the presence of Discharge B in abnormal amounts. The only subject tested with 7 minims of the solution gave a specimen which on addition of heparin produced an abnormally long C.T. The cause of this is not understood, but it suggests that adrenalin is in some way concerned with the action of heparin in the body.

Discharge A.—If a second prick be made within 30 seconds of the taking of a control specimen, and in the same area, the effect of

TABLE VII.

Expt. No.	B.C.T., in minutes, of initial prick.		B.C.T., in minutes, of 2nd prick after an interval of 4 to 5 minutes.	
	Control.	$+2\frac{1}{2}$ parts of heparin.	Control.	$+2\frac{1}{2}$ parts of heparin.
419	$5\frac{1}{2}$	12	$4\frac{1}{2}$	$10\frac{3}{4}$
497	$5\frac{1}{4}$	$11\frac{1}{2}$	5	$9\frac{3}{4}$
539	$7\frac{3}{4}$	6	$5\frac{1}{2}$	8
525	$4\frac{1}{2}$	6	$4\frac{3}{4}$	5

Note.—Expt. 525 exhibits the lengthening effect on C.T. of Discharge A when it acts on blood with a C.T. less than 5 minutes.

Discharge A will not be observed, but if the second test be made after a slightly longer interval, say one minute, it may be found. It is usually observed most readily after about 4 minutes.

The changes in C.T. suggest that either Discharge B merely follows Discharge A, or alternatively, that the two neutralise each other. If neutralisation occurs, then an addition of heparin to blood containing Discharge A should produce less effect than on the same blood prior to the entry of the Discharge A. The results of tests of this kind were as

point of injury where presumably Discharge A is higher. If the first prick be made in a finger or toe shut off from the general circulation the same general rise in C.T. occurs as when no tourniquet is present. The blood samples taken after the initial prick from fingers or toes which were shut off by tourniquets showed a rise in C.T. sometimes exceeding that found outside the tourniquet areas (fig. 11). We therefore believe that Discharge B is produced remotely from the initial prick by nerve action and not by the diffusion of substances from the traumatised area, and also that the discharge can be produced by nerve action in the tissues at the ends of the fingers or toes.

In these experiments the internal temperature would be normal, but it is a constant finding that when the skin surface is relatively cold the amount of Discharge B is small or does not appear at all. We infer that with the trauma used, the skin is the chief source of Discharge B.
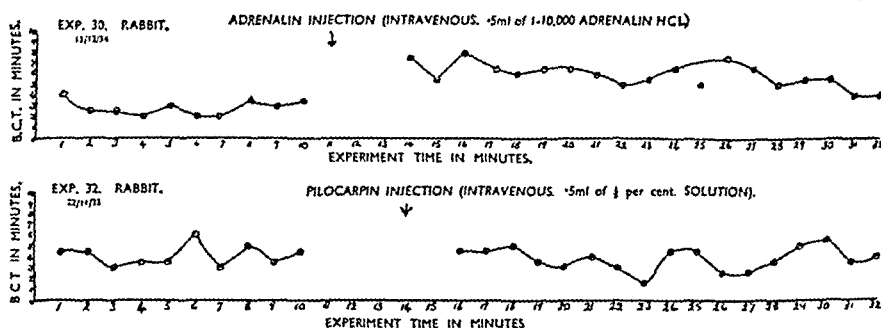

FIG. 10.—The effect of intravenous injections of adrenalin and pilocarpin on two rabbits. Normal C.T. changes are shown in the beginning of each experiment.

The foregoing experiments made it desirable to examine the effect on B.C.T. of stimulating the sympathetic and the parasympathetic autonomic nervous systems, and also the direct effect of acetylcholine and adrenalin on blood *in vitro*. The fact that the production of Discharge B is a general and not a regional effect suggests that adrenalin might be the chemical mediator involved.

Six rabbits were given i.v. injections of adrenalin and six others were given pilocarpin similarly. Blood was taken from the ear veins. In two cases there was a marked trauma effect due to the withdrawal of the blood and in these the adrenalin did not cause much change in B.C.T. In the other adrenalin experiments the results are illustrated by Expt. 30, fig. 10. Here the tracing during the first ten minutes is fairly typical of results obtained in a quiet rabbit. Following the injection of adrenalin there *is a marked and sustained rise in B.C.T.* resembling the effect of Discharge B described in this paper. In the pilocarpin experiments violent oscillations in C.T. occurred, but it does not give the same sustained rise as adrenalin.

The effect of adrenalin hydrochloride on human blood *in vitro* was examined by adding it in quantities to give final concentrations of 1 in 6 millions down to 1 in 40 millions.   It was found that all blood, whatever its initial C.T., and whatever the quantity of adrenalin within the limits quoted, was made to clot in 5 minutes.   In this respect the action of adrenalin is similar to that of Tol. Blue and also to Discharge A.

Adrenalin hydrochloride (1 : 1000) has been injected subcutaneously, in doses ranging from $\frac{1}{2}$ to 7 minims into nine apparently normal human subjects.   The subcutaneous injection of adrenalin is painful and hence will cause the release of considerable Discharge A.   Any effect in other directions will thus have superimposed upon it the effect of this Discharge A.   The changes in C.T. were consistent with the presence of adrenalin or with Discharge A in amounts larger than found in our other experiments.   In none of these cases did the C.T. suggest the presence of Discharge B in abnormal amounts.   The only subject tested with 7 minims of the solution gave a specimen which on addition of heparin produced an abnormally long C.T.   The cause of this is not understood, but it suggests that adrenalin is in some way concerned with the action of heparin in the body.

Discharge A.—If a second prick be made within 30 seconds of the taking of a control specimen, and in the same area, the effect of

TABLE VII.

Expt. No.	B.C.T., in minutes, of initial prick.		B.C.T., in minutes, of 2nd prick after an interval of 4 to 5 minutes.	
	Control.	+ 2$\frac{1}{2}$ parts of heparin.	Control.	+ 2$\frac{1}{2}$ parts of heparin.
419	5$\frac{1}{2}$	12	4$\frac{1}{2}$	10$\frac{3}{4}$
497	5$\frac{1}{4}$	11$\frac{1}{2}$	5	9$\frac{1}{4}$
539	7$\frac{3}{4}$	6	5$\frac{1}{4}$	8
525	4$\frac{1}{2}$	6	4$\frac{3}{4}$	5

Note.—Expt. 525 exhibits the lengthening effect on C.T. of Discharge A when it acts on blood with a C.T. less than 5 minutes.

Discharge A will not be observed, but if the second test be made after a slightly longer interval, say one minute, it may be found.   It is usually observed most readily after about 4 minutes.

The changes in C.T. suggest that either Discharge B merely follows Discharge A, or alternatively, that the two neutralise each other.   If neutralisation occurs, then an addition of heparin to blood containing Discharge A should produce less effect than on the same blood prior to the entry of the Discharge A.   The results of tests of this kind were as

shown in Table VII. In each experiment at the time of the second prick there was a preponderance of Discharge A, and in each case part of the heparin has been neutralised. The effect on the C.T. of blood free from heparin may be very marked, as in Expt. 539, but the change when heparin is present is small. This indicates (*a*) that Discharge A and heparin do neutralise each other, and (*b*) that the amount of Discharge A released in these experiments is small compared with that needed fully to neutralise either $2\frac{1}{2}$ parts per mill. of added heparin, or the Discharge B.

By means of the fall in C.T. and the effect of heparin addition the distribution of Discharge A in the body may be examined. An initial prick was made on a thumb. After $4\frac{1}{2}$ minutes the same thumb was pricked again and simultaneously a finger on the same hand was pricked. To part of each specimen heparin was added with results of which examples are given in Table VIII. It will be seen that (*a*) the C.T. of

TABLE VIII.

	2nd prick to thumb.		Prick on other finger.	
Time of prick.	Control.	+Heparin $2\frac{1}{2}$ parts per mill.	Control.	+Heparin $2\frac{1}{2}$ parts.
$4\frac{1}{2}$ min. after first prick. „	$5\frac{1}{2}$ min. C.T. 6 „ „	$7\frac{3}{4}$ min. C.T. $8\frac{1}{2}$ „ „	$5\frac{1}{2}$ min. C.T. $6\frac{3}{4}$ „ „	9 min. C.T. $13\frac{1}{4}$ „ „

the control is lower from the thumb which had been previously pricked, and (*b*) more heparin has been neutralised in the specimens from the same thumb.

Fig. 11 gives the results of two experiments in which the initial pricks have been made to the left thumbs and tourniquets applied to the middle fingers of the same hands to increase the Discharge A effect. (This effect with the tourniquets is dealt with below.) The normal rise due to trauma appears on the right hands, but, with one exception, it is less in the fingers of the left hands. This exception occurs where a tourniquet has been applied. Experiments, of which these are typical, lead us to the view that Discharge A is produced locally at or near the site of the trauma and then enters the general circulation. The higher C.T. inside the tourniquet area results from the non-entry of the Discharge A, while the nervous mechanism releases Discharge B in that area in the same way as in the rest of the body.

Effect of Tourniquets.—We have made 25 tests in which a tourniquet was applied to the finger to be pricked and then released immediately

after the prick.   In 20 of these the maximum rise in C.T. shown by subsequent pricks to other fingers of the same hand was $1\frac{1}{2}$ minutes (compare with fig. 8).   In 12 similar tests in which the tourniquet was applied to the digit adjoining that first pricked the maximum subsequent rise was $1\frac{1}{2}$ minutes.   These results indicate that increase in the amount
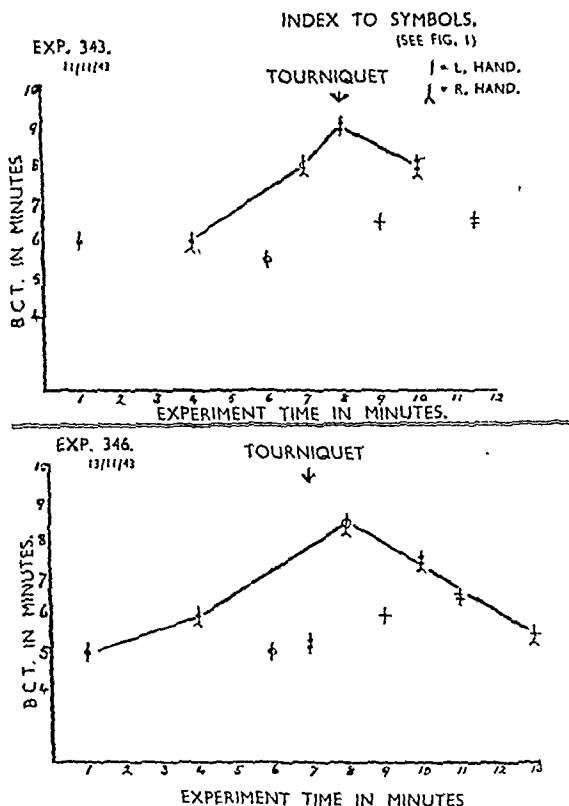

FIG. 11.—Two experiments showing that the effect of Discharge B is less in the hand subjected to the initial trauma, and which also has a finger tourniquet on it, than on the other side of the body.

of trauma leads to increase in the amount of Discharge A, but suggest that the amount of Discharge B does not increase in proportion.

We have made tests to determine the lowest point to which the C.T. can be reduced by Discharge A.   Whether the trauma is increased by pricks or by the application of a tourniquet the maximum effect remains the same as given in fig. 7.   Discharge A, though it reduces C.T., is not a coagulant.

We find that a similar action on C.T. is given by adrenalin and a preparation of histamine base.   The action of Tol. Blue we have already described.   This similarity suggests that Discharge A is a chemically

basic substance, a view which is supported by its interaction with heparin.

DISCUSSION.

The effects of a prick have been previously investigated by Lewis [1927], who found that "H" substance was produced. This may be identical with Discharge A, but we have not proved this. Numerous other workers have investigated the effects of trauma and, though their findings vary, it has been held that histamine, adrenalin, and protein decomposition products are instrumental in causing shock. We have shown that Discharge A, histamine, and adrenalin all combine with heparin, and it is to be expected that heparin will also combine with any other similarly basic substance which may occur in the blood [Fischer, 1935]. If, then, shock be due to a substance or substances of the type mentioned, Discharge B will have an anti-shock function. The fact that it is found only when the skin is warm supports this.

The minimum dose of heparin has been stated by Jorpes [1939] to be 0·25 mg. per kilo of body-weight, *i.e.* approx. 2·5 parts per mill. in the blood. We have shown that an effect may be produced by quantities as small as 0·07 part per mill., but since neutralisation of up to 2·4 parts per mill. may occur, the minimum effective dose must not be less than this amount. When the initial C.T. of the blood exceeds 8 minutes the amount required will be considerably more (see fig. 3).

SUMMARY.

1. It has been shown that the effect on B.C.T. of anticoagulants and other substances, when used in the quantities employed in this work, is dependent on the original C.T. of the blood to which they are added.

2. The effects on blood, *in vitro*, of heparin and Toluidin Blue have been examined in detail; tests have also been made with Chlorazol Fast Pink, adrenalin, and histamine preparations.

3. Reason has been given for the view that heparin is not ordinarily present in the blood. A principle, which is not heparin, has been shown to exist in the blood which can result in wide variations in B.C.T.

4. The amount of anti-heparin substances in the blood has been investigated.

5. The effects of trauma have been shown to include the presence in the blood of two substances which affect B.C.T. and which have been referred to as Discharges A and B.

6. Discharge A is produced locally and probably regionally, and is a chemically basic substance. Its effect on B.C.T. is to reduce it to 5 minutes, but not lower.

7. Discharge B is produced generally, and is of the nature of heparin. It combines with Discharge A, and its flow is intermittent.

8. The view is advanced that, if Discharge A is a shock-producing substance, then Discharge B is the natural antidote to it.

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CONGESTIVE ATELECTASIS IN LUNGS OF RABBITS AND OTHER ANIMALS SUBJECTED TO THE ACTION OF LOW BAROMETRIC PRESSURE.

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WITHIN the last hundred years many workers have described pathological changes found on post-mortem examination of the lungs of mammals subjected to conditions of low barometric pressure. P. Bert [1878], Spehl and Desguin [1909], Heger and de Meyer [1912], Bayeaux [1925, 1927], Campbell [1927], Rosin [1927], Schubert [1930], Hurtado [1932, 1934], Piery *et al.* [1939] observed lung congestion, hæmorrhage, œdema, and emphysema of varying degrees.

Paul Bert studied lung changes in many species after exposure to pressures between 160 and 45 mm. Hg, the decompressions being rapid (3–4 min.). His descriptions of these changes are the clearest and most careful of those recorded by workers in this field. He reported instances of hyperæmia, and of hæmorrhage though these occurred less often, which he considered were not specific for the action of low barometric pressure because similar changes in the lungs were found in animals dying from simple asphyxia. He also confirmed the observation of earlier workers that the lungs of some animals dying as a result of decompression showed large red patches on their surfaces, sank in water but expanded completely when insufflated. He likened this state to that of the fœtal lung and attributed it to the suddenness of decompression. Microscopic examination of the lungs of decompressed guinea-pigs by Hurtado [1934] revealed congestion and patches of atelectasis. His published illustrations suggest an analogous condition to that reported by P. Bert.

Various mechanisms have been put forward as being responsible for the production of the lung hyperæmia. Kronecker [1903] held that the hyperæmia was the direct effect of rarefied air upon the lung capillaries. This view received support from Bartlett [1904], Spehl and Desguin [1909], Heger and de Meyer [1912], and Jacobj [1933], but was strongly

criticised by Zuntz *et al.* [1905] and by Fleisch [1934]. Schubert [1930] suggested that low barometric pressures lead to an increase in the surface tension within the alveoli which in turn causes lung retraction with deformation of the alveoli and passive distension of the alveolar capillaries. In this connection v. Neergaard [1929] pointed out that such alveolar surface tensions are the most important factor in the production of lung retraction.

A decreased vital capacity of the lungs occurs in men living at high altitudes or exposed to low barometric pressure in pneumatic chambers. This finding is explained by Zuntz *et al.* as being due to a displacement of the diaphragm caused by expansion of the intestinal gases, and by Kronecker [1903], Viale [1924], and Schneider [1932] as being the result of congestion of the lungs. Hurtado [1932], however, found that the total lung capacity remains normal, but that there is a reduction in vital capacity which is compensated by an increase in residual air volume. These changes together with congestion suggest an efficient respiratory mechanism for adaptation to low barometric pressures. Schneider [1932] measured the decrease in vital capacity in men exposed to 20,000 feet (349 mm. Hg) in a pneumatic chamber and at 14,000 feet (447 mm. Hg) at Peake's Pike. He found a decrease varying from 6·7 to 15·3 per cent., but when oxygen was added to the inspired air the vital capacity tended to return to its normal level. He concluded that the anoxia caused lung congestion.

This short review of literature indicates that there is no agreement between workers upon the question of whether or not a low barometric pressure exerts a mechanical action on the lungs.

The experiments reported in this paper were designed to determine whether the changes in the lungs found after animals were exposed to low barometric pressure could be ascribed to the concomitant anoxia alone; if some changes were found to be specifically due to low pressure, then the whole problem of its action would have to be carefully investigated.

In this laboratory during the last three years several hundreds of the common laboratory animals were subjected to sub-atmospheric decompressions in the course of an investigation by Drs. Catherine O. Hebb, O. A. Trowell, and others, into some of the physiological problems which arise in such conditions. We were given the opportunity of observing the lungs of many of these animals; our own experimental material included tests on rabbits, guinea-pigs, rats, cats, and one dog.

<div align="center">METHODS.</div>

A decompression chamber of 300 litres capacity was used for the experiments which were carried out, with or without oxygen, at various pressure levels between 275 mm. Hg and 70 mm. Hg. The time taken

for decompression varied from less than a second to several minutes. The animals were maintained at the low pressure level for periods up to 60 minutes and then recompressed.   Sometimes those which survived were killed with nitrogen at ground level, otherwise the survivors were discarded.

The post-mortem examinations were carried out immediately after the experiment.   Before removing the lungs a search was made for gas bubbles in the abdominal and thoracic blood-vessels.   The lungs were examined macroscopically before inflation and when fully inflated.   If specimens were required for microscopical examination they were taken before inflation.

With regard to post-mortem changes, hyperæmia was the most constant lung change found.   It was characterised by a general diffuse redness which appeared to be greatest in the lowest parts of the lungs (according to the position of the animal at death).   This means that the hypostasis in part governed the site of the hyperæmia.   It was classified as mild, medium, or considerable, according to the intensity of the coloration and the area of the lung affected.

Lung petechial hæmorrhages, well-recognised phenomena, and often associated with asphyxial death, were also frequently found.   They were often quite numerous, but sometimes appeared singly on any part of the lung surface.

Lung œdema was observed only occasionally, and was never massive. A certain loss of lung elasticity in some cases indicated some œdema, and histological examination revealed the presence of fluid in the alveoli in isolated cases.

Congestive atelectasis is the term chosen to describe a further lung change in which the microscopical picture showed maximal dilatation of the lung capillaries and complete exclusion of air from the alveolar spaces (fig. 1).   Macroscopically the condition suggested gross hæmorrhage.   The areas affected were sharply limited and stood out from the surrounding tissue because of their liver-like, uniform, dark red coloration (fig. 2);   thus they were readily distinguishable even when they coexisted with severe hyperæmia in the same part of the lung.   Closing the trachea before opening the chest intensified the contrast between hyperæmia and congestive atelectasis.   Inflation of the lung caused the congestive airless parts of the lung to disappear and a restoration of the normal pink coloration, which showed the condition to be congestive and not hæmorrhagic.   The areas affected by congestive atelectasis were not confined to a particular part of the lung surface nor to any one lobe.   However, they seemed to develop more frequently around the hilum or on the dorsal surfaces.   This lung change observed by us appears to be identical with that described by Paul Bert.
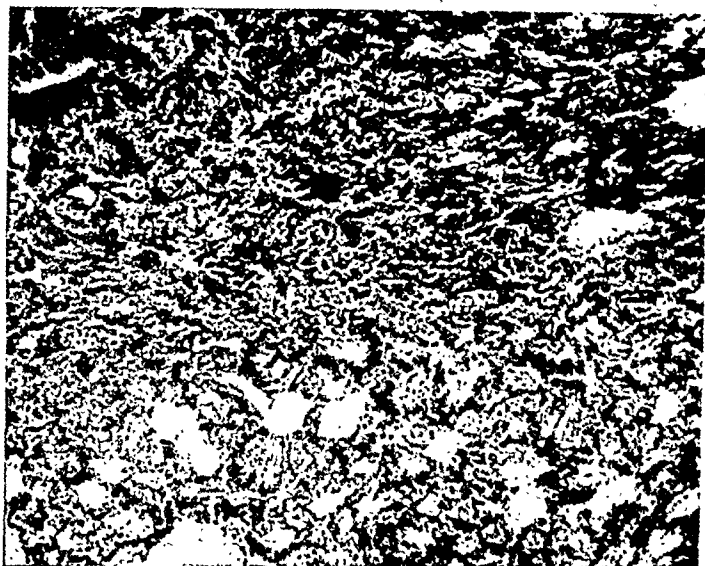
Fig. 1.—Microphotograph (magnification 90) of tissue from a part of the lungs showing congestive atelectasis from a rabbit which died at a level of 71 mm. Hg. *The tissue was fixed by freezing immediately after recompression, and sections 20 μ in thickness were stained with hæmatoxylin and eosin.* Note complete exclusion of air from the alveoli in the upper part of the photograph, contrasted with alveoli containing air below.

Fig. 2.—Photograph of the lungs of a rabbit which died at a level of 100 mm. Hg, taken immediately after recompression. The right lung shows the sharply defined liver-like areas characteristic of congestive atelectasis of the lung. The left lung has been inflated, which has restored its normal appearance, thus illustrating the reversibility of the condition.

I. Description of Experiments designed to provide a Comparison of Lung Changes found in Animals Dying of Simple Anoxia with those found in Animals Dying of Anoxia at Low Barometric Pressure.

Rabbits, 137 in all, weighing between 1·5 and 3·0 kg. were used. They were kept under standard conditions of diet and temperature for one week and fasted 12–14 hours before they were used. The experiments, which were carried out on groups of 4–12 rabbits, confined singly in wire-netted cages in the decompression chamber, were divided into three groups:

(a) Rabbits not subjected to decompression but killed by anoxia at ground level.

(b) Rabbits killed by anoxia while exposed to low barometric pressures.

(c) Rabbits kept at a pressure of several atmospheres, and then killed by decompression to ground level by admission of nitrogen into the chamber.

In groups (a) and (b) the chamber partial pressure of oxygen was maintained at a level critical for survival (20–48 mm. Hg) by adding oxygennitrogen mixtures in the required concentrations via a multiple system of inflow tubes to ensure a uniform atmosphere throughout the chamber. Commercially prepared gas mixtures of different oxygen percentages in nitrogen were used, or corresponding mixtures were obtained by adjusting flows of air and nitrogen through a system of flow meters. The concentration of oxygen in the chamber was determined by analysis of gas samples taken at intervals throughout the experiment. The desired partial pressure of oxygen was reached in about 8 minutes.

In group (b) experiments various subatmospheric pressures from 500 to 100 mm. Hg (10,000 to 48,000 feet) and various speed of decompression (26 seconds to 8 minutes) were tested with the chamber partial pressure of oxygen maintained at 20–48 mm. Hg.

In group (c) experiments a smaller chamber capable of standing a pressure of 6 atmospheres was used, which held only three rabbits at a time.

Post-mortem examinations were carried out as described above.

The results collected in Table I show that in animals dying of anoxia at ground level, hyperæmia of the lungs was a constant finding, but congestive atelectasis did not appear. Similar results were found at barometric pressures between 500 and 300 mm. Hg. At 300 mm. Hg occasionally the animals showed some atelectasis of the lungs, and as the barometric pressure was reduced below this level the incidence of it increased (fig. 3). That the extent of lung surface affected by atelectasis also increased as the pressure was reduced is shown in fig. 4. Fig. 5 indicates that there was no relation between the incidence of

TABLE I.—Lung Changes in Rabbits Dying (a) from Anoxia at Ground Level and (b) from Anoxia during Decompression.

No. of experiment	Atmospheric pressure, mm. Hg	Oxygen partial pressure, mm. Hg	No. of animals	No. of deaths	Hyperaemia of lung					Congestive atelectasis					Frequency of gas bubbles present in vascular system
					Frequency	Intensity				Frequency	Extent*				
						Mild	Medium	Considerable			Slight	Medium	Considerable		
9	760	24·3	8	8	8	2	4	2							
11	760	30·7	8	5	5	3	2								
14	500	41·0	8	2	2	1	1								
3	500	29·0	8	4	4										
17	400	36·0	8	6	6		6								
5	400	32·0	12	8	7										
18	350	32·0	8	3	3		2								
8	300	25·0	5	5	4										
6	300	48·0	6	4	3						3				
19	300	35·0	4	4	4	1	2	4	3						3
20	300	34·0	8	6	3	1	1	3			2	2	1		3
24	300	24·0	4	3	4	1	3	1			1	1			
21	275	32·7	8	6	6			2			2	2			2
23	275	22·0	4	4	4	1		4	2		3	1	3		3
19a	250	30·0	4	4	7	1	2	2	3		1	2	3		3
22	250	32·0	8	8	4	1	3	3			6	1	3		5
25	250	20·0	4	4	6	2	1	2			6	3	1		5
32	200	37·0	8	7	6	1	2	3			6	1	1	2	1
33	150	31·3	6	6	6	1	2	2			4	1	1	2	4
34	150	31·3	6	6	6	1	3	2							
26	100	20·9	4	4	4	1	1	2							

* Slight = 0·1–25 per cent.; medium = 26–50 per cent.; considerable = over 50 per cent. of total surface of the lung.

atelectasis and the chamber partial pressure of oxygen to which the animals were subjected.
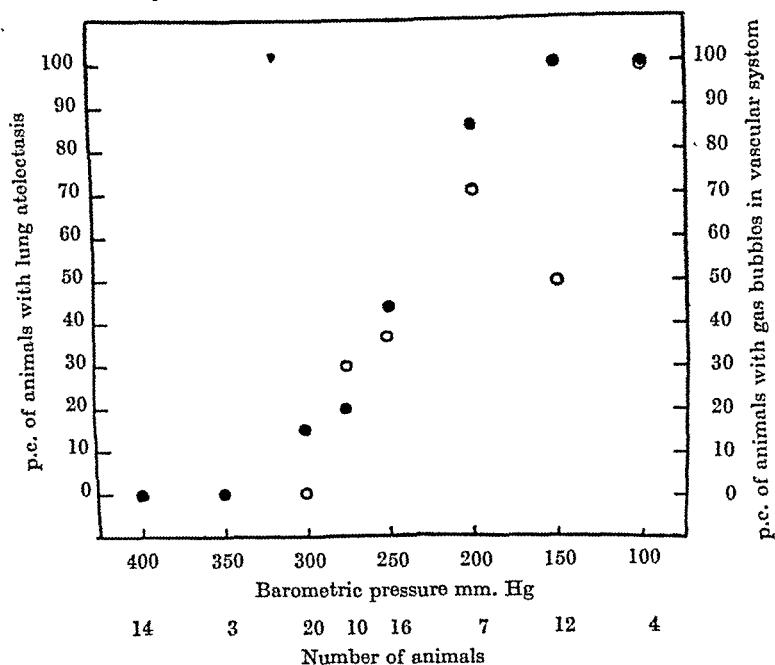

FIG. 3.—The percentage incidence of congestive atelectasis (●) and gas bubbles (O) in rabbits is plotted separately against the barometric pressure level to which they were exposed.

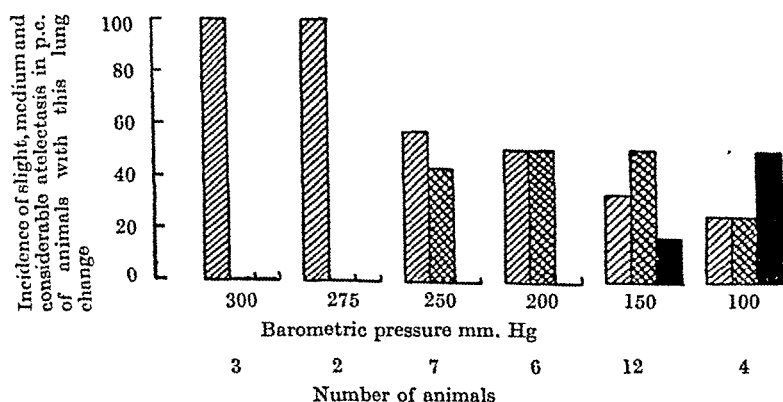FIG. 4.—The incidence of congestive atelectasis, classified according to the amount of lung surface involved, and expressed as a percentage of the total number of animals showing the change, varies with the level of barometric pressure:

slight (not more than 25 per cent. of the total surface);
medium (between 25 per cent. and 50 per cent. of the total surface);
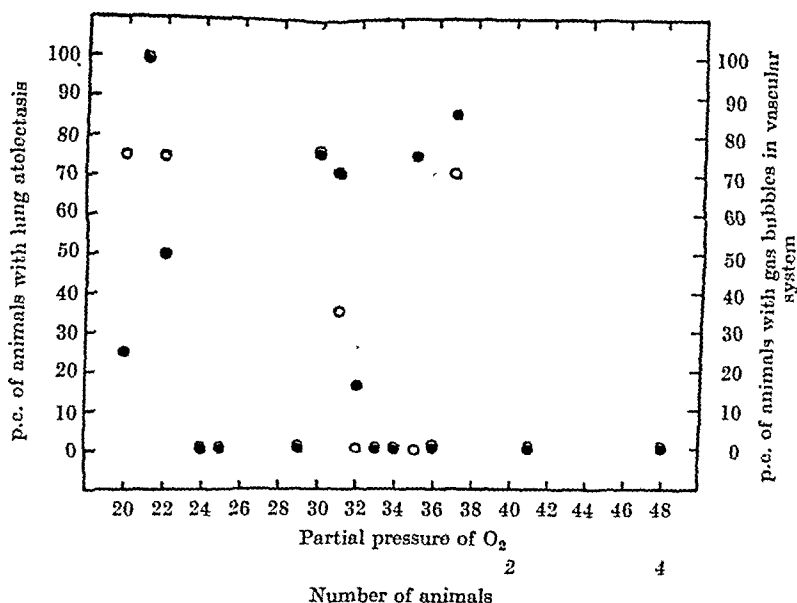considerable (above 50 per cent. of the total surface).

FIG. 5.—Absence of correlation between the percentage incidence of congestive atelectasis (●) and gas bubbles (○) found in rabbits and the partial pressure of oxygen to which they were exposed.

Table II shows that there is no relation between the rate of pressure change (time for decompression) and the incidence and extent of atelectasis.

TABLE II.—INCIDENCE AND EXTENT OF CONGESTIVE ATELECTASIS AND THE RATE OF DECOMPOSITION TO 150 MM. HG (UNANÆSTHETISED RABBITS).

No. of experiment	Time for decompression	No. of animals	No. of deaths	Extent of congestive atelectasis in p.c. of total lung surface				Frequency of G.B. present in vascular system
				0·1–20	21–50	51–70	Over 70	
52	0′ 22″	8	8	1	4	3	0	7
33	0′ 35″	6	6	3	3	0	0	6
48	1′ 20″	4	4	1	0	0	3	4
34	4′ 30″	6	6	1	4	1	..	1
49	38′ 0″	8	8	4	1	2	0	0

Table III shows further that hyperæmia but not atelectasis was found in the lungs of animals subjected to great changes of pressure when decompressed from several atmospheres to ground level.

TABLE III.—EFFECT OF DECOMPRESSION FROM HIGH PRESSURE TO
GROUND LEVEL IN RABBITS.

No. of experiment	Decompression to ground level from the pressure of	Time of death after decompression	Hyperæmia present in No. of animals	Congestive atelectasis present in No. of animals	Gas bubbles present in No. of animals
50	3 atm.	4′	1 : 3	0 : 3	0 : 3
50	5 atm.	7′	3 : 3	0 : 3	3 : 3
50	5 atm.	3′	3 : 3	0 : 3	3 : 3

Gas bubbles were found in animals decompressed from ground level to below 300 mm. Hg or in animals decompressed from 3 to 5 atmospheres to ground level. It is well known that gas-bubble formation is related to the extent and speed of pressure change and that it is not a specific result of low-pressure action. The correlation between the incidence of gas bubbles and decompression level is shown in fig. 3, the correlation between the incidence of gas bubbles and speed of decompression is shown in Table II. That there is no correlation between the incidence of gas bubbles and the chamber partial pressure of oxygen during the time of exposure to low pressure is shown in fig. 5.

It is possible to conclude from these observations that there is evidence of a lung change (congestive atelectasis) formed at barometric pressures below 300 mm. Hg which is not found in animals dying of anoxia at ground level. The development of atelectasis appears to be independent of the degree of anoxia to which the animal is exposed; further it does not appear to be an effect of pressure change such as is necessary for the formation of gas bubbles, but it is dependent upon the absolute rarefaction of the atmosphere.

II. AN INVESTIGATION INTO CONDITIONS RESPONSIBLE FOR LUNG CONGESTIVE ATELECTASIS FORMATION AT LOW BAROMETRIC PRESSURES.

Evidence of atelectasis was also obtained in guinea-pigs, rats, cats (6), and a dog after exposure of less than an hour's duration to low barometric pressures. Further experiments were carried out on rabbits alone in order to isolate if possible the main factors connected with its development, especially whether the appearance of atelectasis depended upon physiological processes or could occur in the dead animal.

1. *Decompression of Dead Animals.*—Six rabbits were killed by nitrogen at ground level and were then immediately decompressed. Heparin was injected intravenously into some of these animals before death as a precaution against intravascular clotting; such clotting, it

was thought, might have prevented any redistribution or displacement of blood caused by the changing mechanical conditions of experiment.

No atelectasis was found in the lungs of these animals (Table IV), though gas bubbles in the vascular system were very numerous. The lungs showed hyperæmia and hæmorrhages, changes found in asphyxial death. It was thus concluded that exposure of the living animal to a condition of subatmospheric decompression was a requisite factor for the development of lung atelectasis in our experiments.

2. *Recompression of Gasping Animals.*—In order to determine the incidence of atelectasis in animals decompressed under the most severe

TABLE IV.—DECOMPRESSION TO 120 MM. HG OF THE RABBITS KILLED BY NITROGEN AT GROUND LEVEL.

No. of experiment	No. of animals	Rate of decompression	Congestive atelectasis present in	Hyperæmia present in	G.B. present in	Remarks
43	4	4′ 45:	0 : 4	4 : 4	3 : 4	Heparin in two.
45	2	1′ 10:	0 : 2	2 : 2	2 : 2	Heparin in one.

conditions compatible with life, rabbits were decompressed individually. At the onset of gasping the animals were killed by recompression into nitrogen or coal gas.

The results of these experiments (Table V) show that animals recompressed while gasping after exposure of short duration to low barometric pressure had lung atelectasis; those animals which respired at normal atmospheric pressure for a certain length of time after recompression did not have it. It was concluded that the death of the animal in a condition of low barometric pressure was not an essential factor for the development of lung atelectasis. Further survival even for the short time after recompression reduced the degree of atelectasis.

3. *Experiments on Anæsthetised Animals.*—The remainder of the experiments was carried out on anæsthetised animals which allowed us more freedom of technique.

Hanson and Sjöstrand [1934/35], working on the mechanism of formation of lung atelectasis in anæsthetised animals (subsequently killed by decapitation), found that there were other factors predisposing to lung atelectasis apart from those generally accepted as of importance, such as blocking of a bronchus with subsequent absorption of air from the corresponding alveoli. They discovered atelectatic areas in the lungs of rabbits and dogs after prolonged urethane or chloralose anæsthesia. Tight bandaging of the chest and abdomen also produced

atelectatic areas, and they were found after a closed pneumothorax, the extent of atelectasis being directly proportional to the duration of the pneumothorax. Hanson and Sjöstrand concluded that any factor which tended to decrease the initial capacity of the lungs might cause atelectasis. We believe that the atelectatic lung changes described by

TABLE V.—RECOMPRESSION OF GASPING RABBITS BY ADMISSION INTO CHAMBER OF NITROGEN OR COAL GAS.

No. of experiment	Atmospheric pressure, mm. Hg	Time of death after recompression	Extent of congestive atelectasis in p.c. of total lung surface	Remarks
42	94	2′	40	Recompressed into nitrogen.
,,	,,	4′ 30″	..	Convulsions after recompression into nitrogen.
,,	,,	1′ 20″	40	Recompression into nitrogen.
44	93	2′ 45″	5	Recompressed into nitrogen.
,,	,,	4′ 40″	0·5	Respiration improved after recompression into nitrogen.
,,	,,	2′	..	Respiration improved after recompression into nitrogen.
,,	148	45″	5	Short period of improved respiration after recompression into nitrogen.
52	150	30″	50	Recompressed into coal gas.
,,	,,	5′	..	Respiration recovered after recompression into coal gas.
,,	,,	5′	..	Respiration recovered after recompression into coal gas.

Hanson and Sjöstrand are similar to those observed by us in animals exposed to low barometric pressures.

The experiments were carried out in which tracheotomised rabbits anæsthetised by intravenous injections of urethane were exposed to pressures ranging from ground level to 102 mm. Hg. The rabbits were immobilised in the supine position. The interval between full anæsthetisation and the beginning of decompression never exceeded forty minutes. The rabbits used for experiments at ground level were kept for the same period of time under anæsthesia before they were killed by nitrogen.

In all further experiments the animals were subjected to the same procedure unless it is specifically stated to be otherwise.

The results of experiments given in Table VI show that anæsthetised tracheotomised rabbits are affected by the same atelectatic lung changes at low barometric pressure as unanæsthetised animals. In anæsthetised animals, however, atelectasis formation, though much reduced in com-

TABLE VI.—Rabbits Anæsthetised with Urethane.

No. of experiment	Atmospheric pressure, mm. Hg	Duration of anæsthesia before death	Time for decompression	Extent of congestive atelectasis in p.c. of total lung surface	Remarks
114/1	Gr. level	25′	..	0	
113/2	,, ,,	36′	..	0·5	
115/1	,, ,,	37′	..	0	
115/2	,, ,,	35′	..	0·2	
57	190	15′ (circa)	5′ 45″	25	
76/1	147	25′ (circa)	3′ 30″	2	
94	120	,,	1′ 35″	30	
95	,,	,,	1′ 45″	20	
110/1	,,	,,	,,	40	
110/2	,,	,,	,,	50	
111/1	,,	,,	1′ 40″	0	Two well-marked hæmorrhagic areas.
111/2	,,	,,	,,	70	
67	117	,,	3′ 40″	30	
60/2	102	,,	4′ 10″	4	
61/2	,,	,,	3′ 30″	70	
38/1	71	,,	0′ 65″	50	
38/2	,,	,,	,,	60	

parison with similar animals exposed to low atmospheric pressures, is not excluded at ground level.

(a) *Experiments in which any Prolonged Response of the Animals to Anoxia before Death was Eliminated.*

A few tests were made in which the rabbits were subjected to atmospheres rich in oxygen (by intratracheal insufflation of pure oxygen) at ground level or at the level of 110–120 mm. Hg for ten minutes and then killed by cyanide. The jugular vein of the rabbit had been cannulated to allow rapid injection of the 2 per cent. potassium cyanide solution, 3–5 c.cm. of which was found to stop all respiratory movements within 20 seconds. In the low-pressure experiments injection was made by a syringe outside the chamber connected by narrow rubber tubing to the jugular cannula of the animal within the chamber. We could not ensure that dissolved gases in the solution might not form intravascular bubbles. As death occurred very rapidly, however, we hoped that the release of bubbles, if it occurred, would not materially alter the results.

The results are shown in Table VII. It can be seen from them that congestive atelectasis formation occurred in the lungs of animals exposed to pressures of 110–120 mm. Hg whose condition previous to the sudden cessation of respiration was good. It was concluded that atelectasis formation at low barometric pressures was not necessarily dependent

TABLE VII.—EXTENT OF CONGESTIVE ATELECTASIS IN LUNGS OF RABBITS KILLED BY KCN AT GROUND LEVEL AND AT LOW BAROMETRIC PRESSURE.

No. of experiment	Atmospheric pressure, mm. Hg	Duration of anæsthesia before death	Time for decompression	Extent of congestive atelectasis in p.c. of lung surface
103/1	Ground level	15' (circa)	..	..
117/1	,,	45'	..	..
117/2	,,	47'	..	..
103/2	119	45'	2' 30"	15
103/3	,,	30'	3' 30"	2
119/1	110	48'	3'	25
119/2	,,	26'	2' 30"	2

upon the response of the animal to critical anoxic conditions prior to its death.

(b) *An Investigation into the Relation of Heart Action to Atelectasis Formation in Lungs of Anæsthetised Animals with Open Chest at Ground Level.*

Experiments at ground level were performed on four rabbits. The chest was opened by removal of the sternum. The lungs were maintained in a semi-deflated state by an inflow of oxygen *via* a tracheal cannula. A description of one of the experiments (No. 104) follows:—

> On touching gently an area near the hilum of the diaphragmatic lobe of the left lung with the handle of a scalpel, the area concerned became very rapidly liver-like and contrasted sharply with the remainder of the slightly hyperæmic lung tissue. The liver-like area spread outwards slowly. This action was repeated on the same part of the right lung and a similar result was obtained. Inflation of the lungs by increasing the oxygen pressure restored to them their normal appearance, which was still maintained when they were deflated to their original position. Then several areas on different lobes of each lung were touched with the scalpel handle and all responded in the manner previously described; meanwhile the heart continued to beat regularly and vigorously. The lungs were inflated once more and the oxygen flow was stopped. When the heart ceased to beat the compression was tried again. This time there was no development of the atelectatic areas. When the oxygen flow was started again and massage of the heart restored its vigorous action, gentle local compression of the lung surface again caused formation of the liver-like patches. We repeated these tests with the heart several times, always obtaining the same result.

The results of this experiment were confirmed on three other rabbits, on which also further qualitative tests were made. Solutions of histamine (1 : 1000) and acetylcholine (1 : 10,000) were applied locally to the surface of the lungs but no atelectasis developed. Forcible displacement of the liver and stomach in · a thoracic direction by manipulation through an abdominal incision resulted in the appearance of atelectatic areas on the diaphragmatic lobes of the lungs. When in this manner direct pressure was applied to the lung surface with the viscus in · various stages of inflation, it was found that the greater the degree of inflation the greater the force required to produce the atelectic areas.

We were convinced that the lung change described above, because of its macroscopic appearance and reversibility, resembled exactly the atelectatic condition which we found in the lungs of animals after exposure to low barometric pressures. From these qualitative experiments at ground level we felt able to conclude that the atelectasis could only occur in animals in which some degree of pulmonary circulation of the blood was maintained. On this evidence we assumed that atelectasis formation at low barometric pressure, with or without anoxia, can only occur in living animals.

(c) *Experiments to show whether Decreased Lung Capacity Influences Atelectasis Formation.*

Tests were carried out at atmospheric pressure and at low barometric pressure (140–120 mm. Hg) on rabbits tightly bandaged from axillæ to pelvis. The length of the time an animal was kept bandaged before dying of anoxia caused by respiring nitrogen varied from 1·30 to 40 minutes in the ground-level experiments. The length of the time animals were kept bandaged before dying of anoxia at 140 or 120 mm. Hg did not exceed 15 minutes. The results of these experiments are given in Table VIII.

TABLE VIII.—INFLUENCE OF DECREASED LUNG CAPACITY ON ATELECTASIS FORMATION AT GROUND LEVEL AND AT LOW BAROMETRIC PRESSURE.

No. of experiment	Atmospheric pressure, mm. Hg	Duration of anæsthesia before death	Time for decompression	Extent of congestive atelectasis in p.c. of lung surface
106/1	Ground level	15′ (circa)	..	..
106/2	,,	,,	..	0·1
107	,,	32′	..	35
108	,,	,,	..	25
109	140·	36′	6′	85
98/1	122	35′	1′ 35″	70
98/2	,,	25′	,,	90

The results of experiments at ground level confirm the observations of Hanson and Sjöstrand which were made on bandaged anæsthetised animals (killed by decapitation). Atelectasis formation was increased in these conditions and was proportional to the duration of the limitation of lung capacity. The results of experiments at 140–120 mm. Hg show that atelectasis formation was found to be more extensive in animals at these levels than at ground level, and more so than in control animals at 120 mm. Hg (See Table VIII).

Bandaging of either chest or abdomen alone, or injection of large quantities of air into abdominal cavity, did not appear to increase atelectasis formation at 120 mm. Hg.

(d) *Investigation into Methods Preventing Lung Atelectasis Formation in Animals Exposed to Low Atmospheric Pressures.*

(1) Experiments in which the increase of abdominal pressure at low atmospheric pressures was reduced.

. A group of tests was carried out on three rabbits whose abdominal wall had been divided from pelvis to xiphoid process; the abdominal contents thus exposed were covered with warm saline pads. One other rabbit had three wide rubber tubes inserted, one into the stomach, two into the large intestine, and its abdominal wall was closed by sutures, allowing protrusion of the rubber tubes.

The results of these experiments given in Table IX show that although animals in which the increase of abdominal pressure is prevented develop less extensive lung atelectasis than the controls (see

TABLE IX.—REDUCTION OF ABDOMINAL PRESSURE.

No. of experiment	Atmospheric pressure, mm. Hg	Time for decompression	Duration of anæsthesia before death	Extent of congestive atelectasis in p.c. of lung surface
88/1	117	1′ 30″	*circa* 20′	1
88/2	,,	,,	,,	5
93/1	122	1′ 40″	30′	10
93/2	,,	,,	20′	3

Table VI), yet under these conditions formation of it is certainly not excluded.

(2) Experiments in which slight positive intra-pulmonary pressure was maintained in animals at low barometric pressures.

Rabbits were used for these experiments. Fig. 6 shows the arrangement by which a positive intra-pulmonary pressure was maintained at any subatmospheric pressure level. The rabbits' tracheal cannula was connected with a side tube. The pressure difference between the

atmosphere outside and within the decompression chamber caused a flow of air or oxygen conveniently regulated by tap (1) outside the chamber. It was possible to keep a steady (except for the small
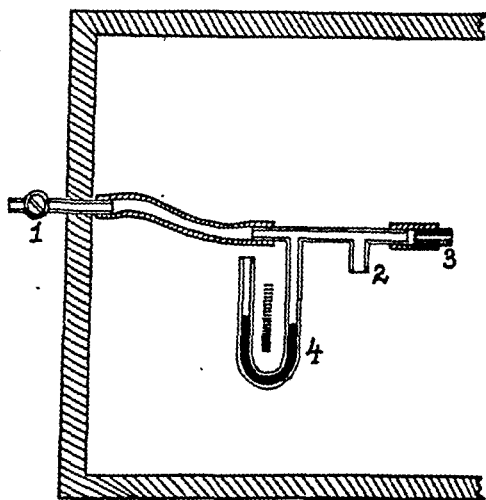

FIG. 6.—Method for maintaining positive intra-pulmonary pressures at any subatmospheric pressure level within the chamber.

    (1) Tap connecting chamber with outside atmosphere.
    (2) Side tube connected to tracheal cannula.
    (3) Capillary tube for resistance.
    (4) Mercury manometer.

respiratory variations) positive intra-pulmonary pressure throughout the experiment in this way.

The results of these experiments are given in Table X and show that maintenance of an adequate positive intra-pulmonary pressure seemed to prevent the development of lung congestive atelectasis at the pressure levels tested. The existence of a relation between the sub-

TABLE X.—INCREASE OF INTRA-PULMONARY PRESSURE AND LUNG ATELECTASIS FORMATION AT LOW BAROMETRIC PRESSURE.

No. of experiment	Atmospheric pressure, mm. Hg	Time for decompression	Intra-pulmonary pressure, mm. Hg	Extent of congestive atelectasis in p.c. of total lung surface
54	150	8′	8	0
70	120	3′ 30″	4	0
69	117	3′ 45″	1	15
58	110	45″	6	0·5
64	109	4′ 30″	5	0·2
63	107	4′ 10″	10	0
62	92	4′ 20″	10	0

atmospheric pressure level and the (minimum) positive intra-pulmonary pressure necessary to prevent atelectasis formation is suggested by the figures reported in Table X.

DISCUSSION.

It has been shown that the atelectasis which occurs in animals exposed to barometric pressure of less than 300 mm. Hg for periods up to 60 minutes is due to the low pressure *per se* and not to the concomitant anoxia. The precise physiological mechanisms producing such atelectasis are somewhat obscure, but we would draw attention to the work of Verzàr and Jeker [1937], who came to the conclusion that a physiological atelectasis may exist during normal resting respiration and disappear with the onset of increased ventilation of the lungs. Again, atelectasis indistinguishable macroscopically from the type we observed under certain circumstances occurs in anæsthetised animals at ground level [Hanson and Sjöstrand, 1934/35]. Thus it may be that at low barometric pressures physiological atelectasis is exaggerated and so persists in spite of the increased lung ventilation which results from the anoxia.

That changes in surface tension within the alveoli [v. Neergaard, 1929] may be brought about by a low barometric pressure has been suggested by Schubert [1930]. He expressed the view that these changes reduced the intra-alveolar forces which normally oppose collapse of the lung. Our experiments do not militate against this view; on the other hand they give no direct support to it. They do, however, indicate the importance of keeping a balance between the pressure of the air within the lung, and the active or passive forces responsible for lung retraction if atelectasis is to be prevented. Thus we found that the maintenance of a small positive intra-pulmonary pressure at low barometric pressure is effective in preventing atelectasis, and that limitation of the forces promoting lung expansion increases the degree of atelectasis.

Our results suggest that positive pressure breathing at high altitudes will be beneficial in maintaining expansion of the lungs.

SUMMARY.

1. In rabbits, guinea-pigs, rats, cats, and a dog subjected to the action of low barometric pressure, congestive lung atelectasis, of varying extent, has been found at post-mortem examination.

2. Experiments on rabbits exposed to decreased partial pressures of oxygen at different barometric pressures have shown that there is no relation between the incidence of congestive atelectasis and partial pressure of oxygen. The animals dying of anoxia at ground level or at barometric pressures between 500 and 300 mm. Hg showed all the usual lung changes but no congestive atelectasis.

3. The animals decompressed from 3 or 5 atmospheres pressure to ground level also did not exhibit this lung change. There was no relation between the rate of decompression from the normal to low barometric pressures and the incidence or extent of congestive atelectasis.

4. A direct relation between the level of decompression (300–100 mm. Hg) and the incidence and extent of atelectasis was found, and it was concluded that the absolute rarefaction of air was the prime cause of congestive atelectasis formation.

5. An increase of intra-pulmonary pressure maintained in animals at low barometric pressures prevented lung atelectasis formation.

6. The results of experiments of Hanson and Sjöstrand on the formation of lung atelectasis under the influence of different factors decreasing lung capacity at ground level are confirmed.

7. The possible mechanism responsible for the lung atelectasis formation under the influence of low barometric pressure is discussed.

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RADIOGRAPHIC STUDIES OF THE EFFECTS OF MEPACRINE ON THE GASTRO-INTESTINAL TRACT OF THE RAT.[1]

By Army Malaria Research Unit, Oxford,[2] and The Nuffield Institute for Medical Research, Oxford.[3]

When the members of the M.R.U., Oxford, began their detailed studies of the pharmacology of mepacrine, it was known that this drug produced in some human subjects symptoms and signs referable, at least in part, to altered activity of the gastro-intestinal tract. The literature on mepacrine did not provide a complete explanation of the alimentary canal upsets produced by the drug, so it was necessary for the M.R.U. to include in its programme a more detailed study than had hitherto been made of its gastro-intestinal effects. It was decided to make a beginning on animals and thereafter to extend the study to man. The choice of animals in war-time was limited, as the only ones available in adequate number were rats. Fortunately (see Discussion), this limitation to a genus which does not vomit proved in the outcome to be an advantage rather than the opposite. The technique used for the investigation, radiography, combined with the introduction of radio-opaque media, was chosen (i) because there would be no surgical interference, or anæsthesia, to complicate one's interpretation of the results, and (ii) because objective records could be obtained at suitable intervals of the state of the whole gastro-intestinal tract. This paper describes the technique of the radiographic investigation, and the effects of various doses of mepacrine on the gastro-intestinal tract of the rat.

TECHNIQUE.

White male rats weighing between 125 and 225 g. were used. Throughout the period of investigation they were fed on a standard diet of dry dog biscuit, with unlimited water. In addition, immediately

following the conclusion of an experiment, the rats were given a special meal consisting of bread, toppings, a small quantity of cod liver oil, and greenstuff. For 24 hours before an experiment they were deprived of food and water. One half-hour before the opaque meal was given, a No. 3 gum elastic catheter was introduced into the stomach and the drug to be tested was administered in solution. The volume of fluid introduced was 0·25 ml. per 150 g. body-weight. Before control examinations the same amount of water was given. The opaque meal was a soft pellet weighing 1·5 g. containing two parts by weight of ordinary dog biscuit ground to a powder and one part of bismuth carbonate with enough water added to form a pellet. Except in a very few cases this meal was eaten a minute or two after it was put in the cage. The rats were given ordinary food and water when all the opaque meal had passed beyond the small intestine or at the end of 12 hours, whichever was the earlier.

Each rat was radiographed every half-hour for the first 3 hours after the meal, then every hour until the 6th hour, and thereafter as indicated until the 12th hour. At 24 hours another radiograph was taken, and if bismuth was still present in the stomach, another at 48 hours. Ilfex (non-screen) film was employed with the following factors:—

Target-film distance	.		$18\frac{1}{2}$ inches.
kV	.	.	.	55
mA	.	.	.	90
Exposure time	.	.		0·08 sec.

During radiography the rat was stretched out in a supine position on top of the film and a ventro-dorsal view obtained (fig. 1). The exposure was made at a moment when respiratory movement was minimal. For screening, the rat was held in the same manner over an under-couch tube and viewed from above. On the whole the rats were easily handled and no anæsthetic was necessary. From 18 to 24 rats were used on each experimental day.

The Normal Radiological Appearance of the Gastro-intestinal Tract of the Rat.

Anatomical Note.—The rat does not vomit, and its gut presents certain anatomical differences from the human gut. The stomach is divided into two parts by a transverse, firm, raised ridge easily identifiable in the gross specimen. The part cranial to the ridge is the fore-stomach and the part between the ridge and the pylorus is divided into antrum and fundus (fig. 2). The fore-stomach has a thin, translucent wall lined by stratified squamous epithelium, and it receives the œsophagus at the most caudal point of its lesser curvature. The wall of the antrum and fundus is much thicker, and the muscular layer more developed, than in
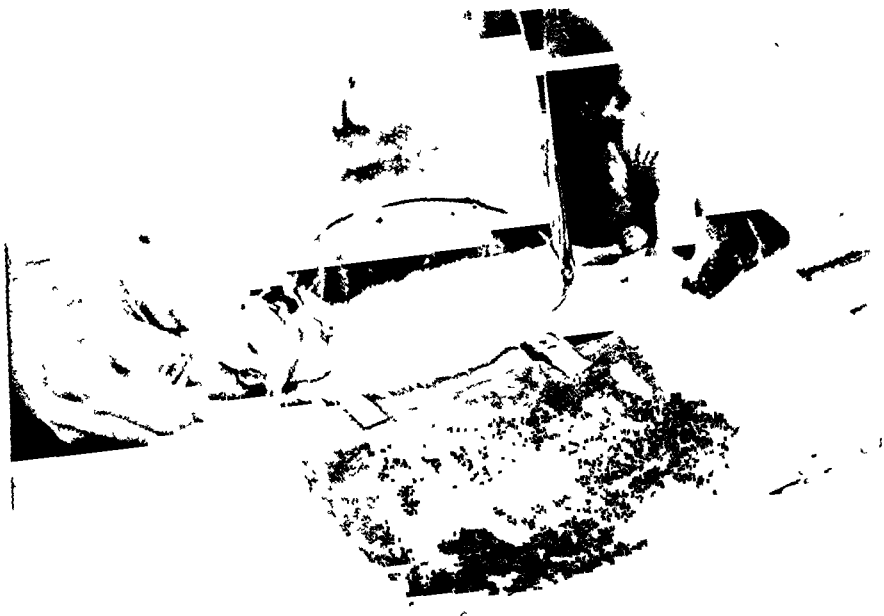
FIG. 1.—The method of holding an unanæsthetised rat over the X-ray film during an exposure.

¼ hr.
Fig 3.

3 hrs.
Fig. 4.

6 hrs.
Fig. 5.

7 hrs.
Fig. 6.

24 hrs.
Fig 7.

¼ hr
Fig 8

3 hrs
Fig. 9.

6 hrs.
Fig. 10.

12 hrs.
Fig. 11.

24 hrs.
Fig. 12.
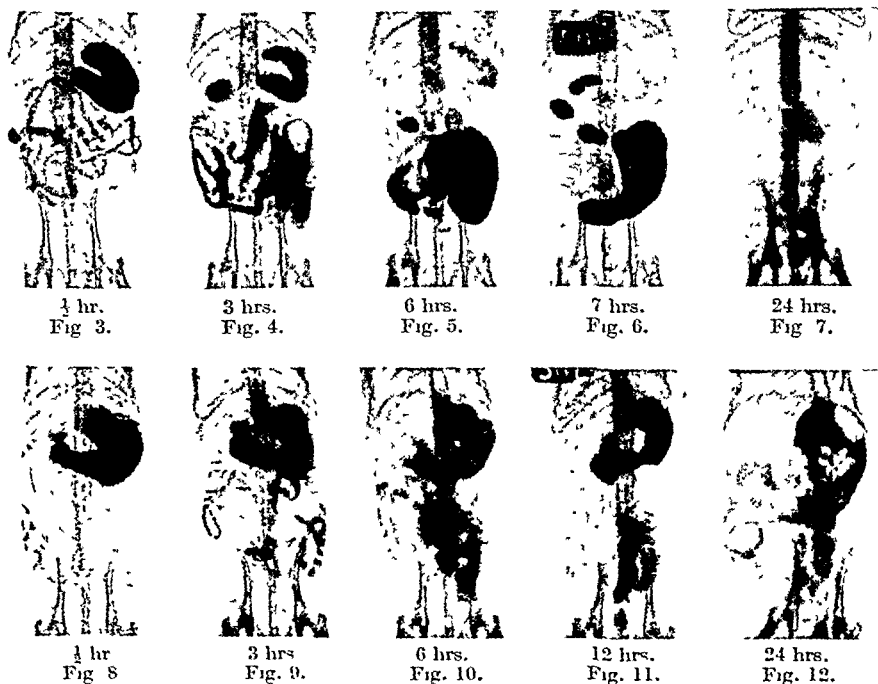
Radiographs of two bismuth meals in the same rat, showing the effect of mepacrine.
FIGS. 3–7.—Control bismuth meal.
FIGS. 8–12.—Bismuth meal ¼ hr. after mepacrine 40 mg./kg.

tlie fore-stomach. In the ridge dividing the fore-stomach from the antrum and fundus the muscular layers are thicker still, but there is no true sphincter. The antrum occupies the entire lesser curvature caudal to the ridge, and the fundus includes the entire greater curvature except for a short distance cranial to the pylorus. The junction between the two is marked by an abrupt elevation of the mucosa. In the antrum the mucosa has a slightly roughened surface, the epithelium is columnar and there are short, relatively wide, mucus-secreting glands. In the fundus the mucosa has a
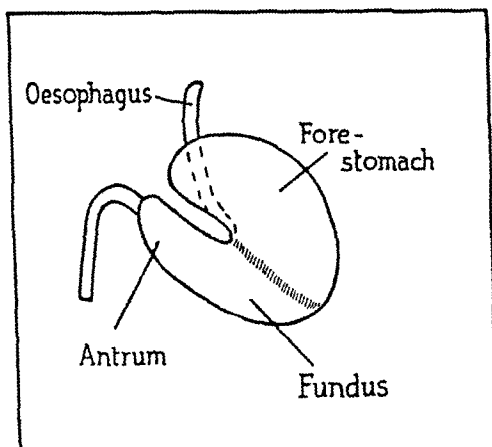

FIG. 2.—Diagram of the stomach of the rat.

furrowed, velvety surface, and the glands are long and narrow and lined by chief and parietal cells.

The rat's duodenum is elongated in conformity with the shape and size of the pancreas. The anatomy of the small intestine is very similar to that of the human small intestine.

The cæcum is several times as wide as the small intestine and varies in length from 5 to 10 cm. It has a long mesentery and its position within the abdomen is much more variable than in man. The colon is about twice as wide as the small intestine and occupies approximately the position it does in man.

The radiographic appearance of the gastro-intestinal tract in a normal rat is shown in Pl. I, figs. 3–7. The stomach is the shape of a horse-shoe with the curved part, the body of the stomach, directed caudally and to the left; the two ends are near the mid-line, the pylorus lying more to the right, however, than the apex of the fore-stomach. The fore-stomach has a greater diameter than the stomach proper, and the outline of its wall is smooth (fig. 3). The junction between the fore-stomach and the antrum and fundus is marked on the lesser curvature by the deepest point of the concavity, and on the greater

animals which gave results around the lower mode came from a different source from the others.   Repeated control meals on a group of 5 animals throughout the period of the experiments.gave very constant results, showing that no change in laboratory conditions had been responsible for the variation found.   If all the work could have been done on one strain of animals, it is probable that the results would have been more consistent.

TABLE I.—MEAN TIMES OF THE PASSAGE OF A BISMUTH MEAL THROUGH THE GASTRO-INTESTINAL TRACT IN 114 NORMAL RATS.

	Mean hours.	Standard deviation.	Standard error of the mean for a group of 6 rats.
Bi first seen in cæcum .	2·87	0·80	0·32
Fore-stomach empty .	4·95	3·76	1·53
Stomach empty .	6·14	3·94	1·61
Small intestine empty .	9·70	4·16	1·70

As well as studying the variation between numbers of normal animals, we have investigated the variation in a group of 5 animals which were given seven control meals at intervals of seven to ten days. Analysis of these results has shown that there is more variation in a group of animals which have a bismuth meal on the same day than there is in the results of a series of bismuth meals in the same animals on different days.   The results are set out in Table II, and the analysis

TABLE II.—EMPTYING TIME OF THE STOMACH IN 5 RATS WHICH HAD 7 CONTROL BISMUTH MEALS.

Rat No.	Control bismuth meal. Emptying time in hours.							Total.
	1.	2.	3.	4.	5.	6.	7.	
1	1·75	3·50	2·25	5·50	3·50	3·50	3·50	23·50
2	1·75	1·75	3·50	4·50	3·50	2·75	2·75	20·50
3	2·25	2·25	3·50	4·50	3·50	3·50	2·75	22·25
4	5·50	6·50	6·50	7·50	9·00	5·50	4·50	45·00
5	4·50	5·50	3·50	3·50	2·75	5·50	3·50	28·75
Total	15·75	19·50	19·25	25·50	22·25	20·75	17·00	140·00

curvature sometimes by a small indentation and at other times by irregularities in the outline of the wall of the fundus which are in contrast with the even outline of the fore-stomach. These irregularities in the shape of the antrum and fundus are due to peristaltic waves which appear immediately after the meal is eaten, and continually change the size and shape of this part of the stomach.

In the normal animal the movements of the fore-stomach are not great enough to be detected on screening; visible peristaltic waves appear on the greater curvature of the fundus just caudal to the fore-stomach and progress slowly towards the pylorus, increasing in depth and indenting the distal part of the lesser curvature. The pylorus opens a minute or two after the meal reaches the stomach and a small amount of food passes through. Thereafter it opens intermittently at fairly frequent intervals. As the stomach empties, the fore-stomach diminishes in size, loses its regular outline (fig. 4), and usually empties one hour before the antrum and fundus. Complete emptying of the entire stomach takes on the average about six hours (see Table I and fig. 6).

As in the human subject, the duodenum is seldom completely filled, and the usual shadow is irregular and thin. There is no duodenal cap. The bismuth meal passes fairly quickly through the jejunum and ileum, and the cæcum is reached in about two and a half hours (fig. 5). The small intestine does not usually fill enough to give a completely opaque shadow, and the column of bismuth becomes fragmented in its passage to the cæcum. Changes in diameter due to movements in the wall tend to be gradual rather than abrupt. In about nine hours the small intestine is empty (fig. 6).

In the rat the colon seldom fills with a solid column of bismuth as in man. Instead, the opaque meal appears in the colon first as a speckling of fæcal masses already present, and later as completely opaque fæcal masses. Fæces with bismuth on the surface may appear in the rectum in four or five hours, but fæces derived from the opaque meal itself take two to five hours longer. By the end of twenty-four hours the entire tract is usually free of bismuth (fig. 7).

Variation in the Rate of Passage of a Bismuth Meal through the Gastro-intestinal Tract of Normal Rats.

As a preliminary to our experiments with mepacrine, the rate of passage of food through the gut was investigated in 114 normal rats. The mean times taken for the standard meal to pass through the stomach and small intestine, and into the cæcum, are shown in Table I. As the standard deviations show, there was considerable variation. Part of the reason for this degree of variation was the fact that animals were obtained from more than one source. The frequency distribution curve of the times taken for the stomach to empty is bimodal, and the

in the small intestine, the cæcum, and the colon are unimportant compared with the effects on the stomach.

Stomach.—The delay in gastric emptying, the most obvious of the effects on the stomach, is the result of pylorospasm and a disturbance of the motility of the body of the stomach.   On screening, no movement is seen in the walls of the fore-stomach, but the entire wall of the antrum-fundus has a greatly increased tone and shows rings of spasm.   In the films these sometimes give the appearance of peristaltic waves, but no orderly progression to the pylorus is seen on screening.   A constant feature of the effect of mepacrine is the marked spasm at the junction of the fore-stomach and antrum-fundus, which in the first few hours makes the division between the two more obvious than in the normal animal (figs. 8 and 9), and later often results in the retention of part of the meal in the fore-stomach after the antrum-fundus is practically empty (fig. 11).   The delay in the emptying of the fore-stomach is a measure of the hypertonicity and spasm of the antrum-fundus and the pylorus.   Once the antrum-fundus loses its hypertonicity and the pylorus is no longer spastic, the meal passes rapidly out of the entire stomach, and there is usually no retention—as there is in the normal animal—of part of the meal in the antrum-fundus after the fore-stomach is empty.

There are other minor features of the effect of mepacrine on the stomach.   There is evidence that gastric secretion is increased.   The size of the gastric shadow is often larger than in the control, and in cases where pylorospasm has been complete, the shadow has increased in size over a period of several hours.   As the volume of the stomach is the volume of its contents, and the bismuth meal is always the same size, the increase must be due to an outpouring of secretion.   Often, too, gas accumulates and a large bubble forms in the stomach (fig. 9).

Small Intestine.—In the great majority of experiments the only change in the radiographic appearance of the small intestine is the result of the disturbed emptying of the stomach.   Occasionally the intestine is much narrower than in the control and it has a thin ribbon-like appearance.   Filling is often irregular, and the bismuth column is more fragmented than it is normally, but this can be explained by the intermittent pylorospasm.   In a few cases there is evidence of an independent delay in the passage of the meal through the small gut; the difference between the time when the stomach is first empty and the time the small intestine is empty is greater after mepacrine than in the control.

Cæcum and Colon.—In a few animals, a large dose of mepacrine has been followed in from two to three hours by a short period of diarrhœa.   The intestinal hurry manifested by the diarrhœa is of short duration, for it has never been reflected in unduly rapid passage of the head of the bismuth meal, *i.e.* the movements of the cæcum and colon

of variance in Table III. There is no significant difference in the variance from day to day in the same animal and the residual variance. There is, however, a significant difference in variance in different animals on the same day and the residual variance ($z=1.22$, $z=0.72$ for the 1 per cent. point). The variation between rats is so much

TABLE III.—ANALYSIS OF VARIANCE OF RESULTS IN 5 RATS WHICH HAD 7 CONTROL MEALS.

	Degrees of freedom.	Sum of squares.	Mean variance.
Columns (variation from day to day) .	6	12·75	2·125
Rows (variation between rats) . .	4	57·02	14·255
Residual . . : . .	24	28·60	1·1917
Total	34	98·37	

greater than that between different meals in the same rat that there is no doubt that more precision is obtained when the mean emptying times in the same group on different days are compared than when the comparison is between the mean emptying times of two groups of rats on the same day. Thus, if R rats are compared with the same group of R rats tested on a different day, the S.E. of the difference of the means of the groups is $\sqrt{\dfrac{2.756}{R}}$, whereas if R rats are compared with a second group of R rats on the same day, the S.E. of the difference of the groups is $\sqrt{\dfrac{6.116}{R}}$. By the first method the number of observations required to establish the significance of a given difference is approximately halved.

The Effects of Mepacrine on the Gastro-Intestinal Tract of the Rat.

The radiographic changes seen in the gastro-intestinal tract of the rat after a large dose (40 mg./kg.) of mepacrine by mouth are shown in Pl. I, figs. 8–12. The most striking difference from the control meal on the same animal (figs. 3–7) is the delay in gastric emptying. Whereas in the control the stomach was empty at 6 hours except for mucosal markings, after mepacrine there was a large amount of bismuth in the stomach at 24 hours. The delay in gastric emptying is related to the dosage of mepacrine (see below), and it may be used to measure the effect of different doses and to measure the effect of variation of adjuvants and vehicles. This phenomenon has been the basis of the quantitative analysis of our experimental results. The changes seen

animal showed a marked effect, and the mean delay was more than 30 hours. The effect of 35 mg./kg. was of intermediate severity. The delay in gastric emptying time caused by these different dosages of mepacrine is given in Table IV.

TABLE IV.—EFFECTS ON THE EMPTYING TIME OF THE RAT'S STOMACH OF VARIATION IN THE DOSAGE OF MEPACRINE.

Dose of mepacrine, mg./kg.	No delay.	½–2 hours delay.	2½–5 hours delay.	6–12 hours delay.	12–24 hours delay.
2·5	2	3	..	..	..
5	2	3	..	..	..
10	1	4	..	..	..
15	3	8	2	7	5
20	1	3	..	1	1
30	1	1	1	2	1
35	..	..	..	3	3
40	..	..	..	..	6

To see if the results could be reproduced, 15 animals were given mepacrine 15 mg./kg. on two occasions. In 5 animals the two experiments were separated by two weeks; in 5 they were separated by four weeks; and in 5 by eight weeks. The results showed very good agreement. The distribution of the differences was the same in each of the three groups, and the mean difference was − 1·28 hours, S.E. 3·47. It should also be pointed out here that throughout the entire period of the investigations into the effects of different dosages of mepacrine, repeated control bismuth meals were given to a group of 5 rats, and that the results of these control meals showed very good agreement.

DISCUSSION.

These experimental results have formed the basis of a comprehensive investigation of the actions of mepacrine on the gastro-intestinal tract. Experiments which defined the gastro-intestinal effects of various salts of mepacrine and of mepacrine dihydrochloride administered in various ways will be described in a further report. The experiments in man which confirmed the important and practical results of the experiments in rats will also be the subject of a further report.

In the present investigation, when mepacrine dihydrochloride was given in a dosage of the same order as the suppressive dosage in man (2–5 mg./kg.), only a proportion of animals were affected and the effects were minimal. It is to be emphasised that the serious effects seen with higher dosages exceed anything which has been seen in man except in special circumstances. The effects of these higher dosages were studied so that data over a wide range would be available for

apparently return to normal before the opaque material reaches them. In some animals there is much more gas in the cæcum after mepacrine than there is in the control, and this accumulation is most marked in those animals' which have a large gas bubble in the stomach. Aside from a few examples of diarrhœa, and the more frequent accumulation of gas, no abnormality has been observed in the cæcum or colon. However, frequent films have not been taken at the time when changes in the large bowel should be looked for after a bismuth meal (12–24 hours), and it may well be that some abnormality has passed undetected.

Effects on the Emptying Time of the Stomach of Variation in the Dosage of Mepacrine.

As has been pointed out, the most obvious effect of mepacrine on the gastro-intestinal tract of the rat is the increase in emptying time of the stomach. As the increases in the different animals can be readily determined, they provide a basis for a quantitative description of the results. For the purposes for the present report, the delay in emptying after mepacrine has been measured by taking the time when the stomach is free of bismuth and comparing it with the time previously observed during the control meal. For example, if the stomach was empty in 12 hours after a dose of mepacrine, and in the control meal it was emptied completely in 3 hours, the delay in final emptying has been 9 hours. There are other ways of measuring the delay in emptying which give an indication of the speed at which the bismuth meal passes through the pylorus at different periods, say from 1 to 3 hours and from 6 to 12 hours, and one of these will be used in a later report. The method used in the present study is simpler, however, and it has been found an adequate method for the present purpose, viz. the measurement of the effects of different dosages of mepacrine.

Observations have been made on the effect of mepacrine dihydrochloride given by mouth in dosages ranging from 2·5 mg./kg. upwards. (Mepacrine 0·1 g. in a man weighing 60 kg. is a dosage of 1·6 mg./kg.) Sixty-four animals have been used for this experiment, with a group of at least five on each dose. Each animal had previously had a control bismuth meal. No change in the general appearance or behaviour of the animal was noticed in any of the experiments. A few animals had a short period of diarrhœa during the first few hours after receiving the drug, but this was unusual.

When mepacrine was given in dosages up to 10 mg./kg. there was delay in emptying of the stomach in only two-thirds of the group, and when there was delay it was slight and the mean delay was about one hour. With dosages of 15 mg./kg. 90 per cent. of the animals showed some effect, and with these dosages the effect was much greater, the mean delay being about 9 hours. With a dosage of 40 mg./kg. every

SUMMARY.

1. A technique has been developed with which the effect of drugs on the gastro-intestinal tract may be studied in the intact unanæsthetised animal. The technique consists in giving rats bismuth meals and observing their rate of passage radiographically.

2. The effects of mepacrine on the rat's gut are: hypertonicity and disturbed peristalsis in the stomach; pylorospasm, increased gastric secretion; delay in gastric emptying; increased accumulation of gas in the stomach and the cæcum; and slight delay in the rate of passage of the bismuth meal through the small intestine.

3. The severity of the effect on the stomach may be measured by determining the length of time taken for the stomach to empty.

4. The effect of various doses of mepacrine dihydrochloride given before the barium meal has been determined, and this information will serve as a basis for comparative studies with other salts of this drug and with other methods of administration.

comparative studies and not because they resemble in degree the disturbances seen in man.

The novel method which has been used has made possible the study of the gastro-intestinal toxicity of mepacrine on a scale which is impracticable in man and deserves comment. Errors due to the variability of the animals have been minimised by standardising the conditions of the experiment, by working with a large number of animals, and by submitting the results to statistical analysis. Only by working with rats was it possible in the prevailing circumstances rigidly to standardise the conditions and use the numbers of animals large enough to obtain statistically significant results. The analysis of the results has shown that during the period of the experiment there was no variation in laboratory conditions which affected the behaviour of the gastro-intestinal tract of normal rats. The variation between strains has been controlled by doing control meals in all animals. The results have also shown that there was no variable in the experiment which affected the response to mepacrine. In spite of the vagaries of the biological material the method has produced statistically significant results.

The view that results of animal experiments cannot be applied to man will be discussed in further publications. It is sufficient for the moment to make three points. First, changes similar in nature to those described for the rat have been demonstrated in man. Secondly, many drugs known to have an effect on the gastro-intestinal tract in man have been tried in rats, and in every case it has been possible to demonstrate some effect radiographically. Details of these experiments will be published shortly. Thirdly, the observations have been made on the intact unanæsthetised animal, and therefore have a great advantage over experiments performed with isolated preparations or on anæsthetised animals.

It seems strange at first sight to choose an animal which does not vomit for use in a study of the pharmacology of drugs which have an emetic action in man. Some of the advantages of working with the rat have already been mentioned, but probably its greatest merit is just this fact that it does not vomit. In animals which vomit, man for instance, the result of an emetic dose of a drug is merely a positive or negative one, and cannot be more than roughly quantitative; the animal vomits, empties its stomach and further observations are impossible. If the dose is sub-emetic, and observations are made radiographically, the result can be measured in several ways, one of which is the speed of gastric emptying. In the rat every dose of an "emetic" drug is sub-emetic, and quantitative observation of the change is possible with any dose. This makes possible a degree of measurement and comparison of results unobtainable in animals which vomit, and thus the characteristic which might be thought to veto the use of the rat becomes its great advantage.

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